MECHANISMS OF NEUROPROTECTION MEDIATED BY GHRELIN IN NEURONAL CELLS

by

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This thesis was prepared under the direction of the candidate’s thesis advisor, Dr. Nicholas J. Quintyne, and has been approved by the members of her/his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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Ghrelin is a 28-amino acid peptide secreted by the stomach. Over the past decade, studies have shown ghrelin to have broad effects on growth hormone release, appetite regulation, and glucose metabolism. These effects are explained largely by the high expression of the ghrelin receptor (GHS-R1a) in both the hypothalamus and pituitary. Recently, ghrelin has been shown to have possible neuroprotective effects in other brain regions expressing GHS-R1a, specifically stimulating anti-apoptotic and anti-inflammatory pathways. The endoplasmic reticulum (ER) is a cellular organelle responsible for processing proteins. When an abundance of misfolded proteins accumulate in the ER, cellular stress pathways ensue, and are linked to a number of neurodegenerative diseases. This project will examine the effects of ghrelin during ER stress in a human neuronal cell line. Expression of genes and proteins involved in potential neuroprotective pathways will be examined to learn how ghrelin mediates its anti-apoptotic effects in neurons.
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**Introduction**

The body secretes small molecules called growth hormone secretagogues (GHSs) in order to stimulate the release of growth hormone (GH). Twelve years ago, ghrelin, a GHS, was discovered and would prove to have a broad effect on stimulating growth hormone, appetite regulation, energy conservation, glucose metabolism, pancreatic function, and immune function, all of which are affected by the aging process (1-5). Furthermore, there has been recent evidence that ghrelin has neuroprotective effects, which could be promising in combating neurodegenerative disease.

Ghrelin is a 28-amino acid peptide hormone produced predominantly by the stomach and activates a G-protein-coupled receptor (GPCR) known as the growth hormone secretagogue receptor type 1a (GHS-R1a) (1, 6). The GHS-R1a is expressed in the hypothalamus, heart, lung, pancreas, intestine, and adipose tissue (1). Studies have shown that the receptor expression has the highest concentration within the pituitary and hypothalamus, but recent data have shown that the receptor is expressed in extra-hypothalamic neuronal populations, which suggests that ghrelin has a larger role in neuronal function (7-9). There are two forms of ghrelin that are found in the bloodstream: acylated (ghrelin) and des-n-octanoyl ghrelin (des-acyl ghrelin) (10). Both forms originate from the proteolytic cleavage of pre-proghrelin and pro-ghrelin, but differ by a posttranslational acylation. The acylation of proghrelin occurs when an octanoic acid is esterified to the Ser3 residue of the peptide (10, 11). This unique modification is carried out by the ghrelin O-acyltransferase (GOAT) enzyme, which precedes the cleavage of proghrelin, the final step in ghrelin activation. (Figure 1) (12, 13).
Figure 1. Structure of both des-acyl and acylated ghrelin found in blood plasma. Posttranslational acylation is done by adding either n-octanoic acid or n-decanoic acid to the 3-Ser residue with the enzyme GOAT. The acylation is needed for ghrelin to activate the GHS-R1a receptor. There is no known receptor for des-acyl ghrelin (14). (Figure from Andrews 2011).
The acylation is needed for ghrelin to bind and activate the GHS-R1a (1, 15, 16). The des-acyl form of ghrelin was initially considered to be a non-active molecular form that had no known function, but recent studies suggest that it may have an impact on appetite and other pathways independent from the GHS-R1a (10). Des-acyl ghrelin accounts for over 90% of circulating ghrelin and has no known receptor. Although there is a large amount of research in progress, it is still unclear what physiological role des-acyl ghrelin has in the body (10).

GHS-R1a is the only ghrelin receptor that has been categorized to be functional. It is a G protein coupled 7-transmembrane receptor that has been identified in the pituitary and hypothalamus in humans (6, 17). The binding of ghrelin to GHS-R1a leads to a change in the transmembrane α helices, which causes a conformational change that promotes the release of guanosine diphosphate (GDP) bound to the G protein α subunit and its exchange for guanosine triphosphate (GTP) (18). This exchange leads to the activation of the intracellular molecules responsible for activating various effector molecules (18). The function of the activated GHS-R1a is to regulate intracellular calcium mobilization activated by phosphatidylinositol- specific phospholipase C (PI-PLC) through a Gαq/11 protein (Figure 2) (6, 19). Other major pathways that are affected by the activation of the GHS-R1a include the mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) pathways (20-23).

The role of ghrelin in the brain has been a topic of interest in the past decade. Although the stimulatory effects of ghrelin on appetite and growth hormone release are mediated through the pituitary and hypothalamus, recent data have shown that GHS-R1a mRNA is highly expressed in the dentate gyrus of the hippocampus as well as in the
substantia nigra (SN) and the ventral tegmental area (VTA) of the midbrain (8). The SN is of particular interest in Parkinson’s disease (PD). PD is the second-most common neurodegenerative disease, affecting roughly 2% of the population who are over 65 years old, and is characterized by the degeneration of dopaminergic neurons, specifically affecting those found in the SN (24). Degeneration of these neurons is the cause of the debilitating motor symptoms (resting tremor, bradykinesia, rigidity, and postural instability) characteristic of PD. GHS-R1a is expressed in dopaminergic neurons in the SN and biotinylated ghrelin has been shown to bind to SN neurons (8, 9, 25). It has also been shown that ghrelin activates SN dopaminergic neurons, which increases the expression of the enzyme tyrosine hydroxylase, thus leading to the biosynthesis of dopamine in midbrain (25). Studies analyzing the effects of ghrelin using PD animal models have yielded extremely interesting results. Using the chemical 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), one can induce a phenotype physiologically similar to PD phenotype in mice (26, 27). The addition of ghrelin showed that dopaminergic neurons were protected against MPTP neurotoxicity via an uncoupling protein 2 (UCP2)-dependent mitochondrial mechanism (Figure 3) (25, 28). UCP2 is a critical mitochondrial protein that regulates reactive oxygen species (ROS) levels, enhances mitochondrial respiration, and increases mitochondrial biogenesis, which contributed to the neuroprotective effects of ghrelin (Figure 3) (29-31).
Figure 2: Proposed signal transduction pathway used by ghrelin to trigger intracellular calcium mobilization. Ghrelin activates GHS-R1a, which activates phosphatidylinositol-specific phospholipase C (PI-PLC), which activates inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) through phosphatidylinositol 4,5-diphosphate (PIP$_2$) with liberation of Ca$^{2+}$ from IP$_3$-sensitive stores and activation of protein kinase C (PKC). PKC inhibits the K$^+$ channels, causing a cellular depolarization, which opens both L- and T-type Ca$^{2+}$ channels. It should be noted the PKC is involved in many other subsequent pathways that include the activation of ERK 1/2 (18, 32, 33) (Figure from Camina 2006).
Ghrelin has also been shown to protect dopaminergic neurons by blocking microglial activation (34). Microglia are support cells responsible for the pro-inflammatory response to stress within the brain. Inflammation plays a significant role in dopaminergic neurodegeneration in the animal MPTP model and human PD (35-40). There is an inhibition of microglial activation and affiliated release of pro-inflammatory cytokines in ghrelin-treated animals after MPTP, thus showing support for ghrelin’s neuroprotective effects in the PD model (34). Several other mechanisms involved in dopaminergic neurodegeneration in PD, including apoptosis, oxidative stress, and mitochondrial dysfunction may also be attenuated by ghrelin (41-43).

Apoptosis is programmed cell death, and ghrelin has been shown to inhibit this process in neuronal cells during oxygen-glucose deprivation (44). The antiapoptotic effects of ghrelin were dependent on the activities of the MAPK, phosphoinositide 3-kinase (PI3K), protein kinase C (PKC), and protein kinase A (PKA) signaling pathways (Figure 3) (44). Apoptosis is driven by a variety of factors, but ghrelin specifically has an effect on the Bcl-2 family of proteins, inhibiting cytochrome c release and caspase-3 activity, while promoting the survival of the neuronal cell. The Bcl-2 family proteins have a major role in the intracellular apoptotic signaling pathway by regulating the permeability of the mitochondrial membrane (45). An increase in permeability of the mitochondrial membrane leads to the release of cytochrome c, which initiates the activation of the pro-apoptotic caspase activation cascade, including caspase-3, -2, -6, -8, and -10 (46, 47). The proapoptotic protein Bax eliminates mitochondrial transmembrane potential ($\Delta \Psi_M$), which promotes the release of cytochrome c, whereas the antiapoptotic protein Bcl-2 conserves the membrane potential and blocks the progression of apoptosis.
Ghrelin increased the Bcl-2 protein levels in cultured hypothalamic neurons, which stabilized the $\Delta \Psi_M$ and inhibited apoptosis in an oxygen and glucose deprived environment (Figure 3) (44). Ghrelin also activates the MAP kinase ERK 1/2 pathway, which is linked to survival pathways, whereas the MAP kinases JNK and p38 are related to neuronal apoptosis (Figure 3) (49). Ghrelin has also been shown to reduce ROS production, which consist of chemically-reactive molecules that can significantly damage cell structures and have an important role in neuronal apoptosis (Figure 3) (44).
Figure 3: Proposed neuroprotective mechanisms of ghrelin. Activation of various kinase signaling pathways (Erk 1/2, Akt 1/2, PI3K, and PKC) leads to the inhibition of apoptotic events. This inhibition is a result of a subsequent increase in the Bcl-2: Bax ratio, prevention of cytochrome C (Cyt), and inhibition of capase-3 (Casp 3) activation (32, 44, 50-52). Ghrelin also prevent activation of proapoptotic events including the activation of p38 and JNK (44). UCP2 is activated by ghrelin, which enhances neuroprotection by suppressing ROS and promoting mitochondrial biogenesis (25, 34, 44, 51, 53). Reduction in ROS also prevents Cyt release. (Figure adapted from Andrews 2011).
A less well-characterized pro-apoptotic pathway originates from the endoplasmic reticulum (ER) and also leads to the activation of the caspase cascade (54-56). The endoplasmic reticulum is a cellular organelle that is responsible for the maturation and processing of proteins through the secretory pathways. As proteins are folded within the ER, there is a constant production of misfolded proteins (41, 57). The appearance of misfolded proteins can activate a protein stress response, called the unfolded protein response (UPR), which increases the production of properly folded proteins, decreases the unfolded protein load, and is connected with inflammatory pathways (58). Although this protective pathway may work for a short time, if the ER is under prolonged stress, the pathway will not be able to overcome the cellular stress and the activation of apoptotic pathways will ensue (59-61). The importance of these protein misfolding pathways has been recognized in recent years, as studies have shown a link between the accumulation and aggregation of misfolded proteins and neurodegenerative diseases including Alzheimer’s Disease (AD), Huntington’s Disease (HD), amyotrophic lateral sclerosis (ALS), prion protein disease, and PD (59, 60). Furthermore, ER stress markers have been reported from post-mortem tissue of the SN dopaminergic neurons in cases of human PD (62-64).

To model ER stress in vitro, chemicals that disrupt the normal processes of the ER can be utilized. Thapsigargin (Figure 4), is a chemical that blocks the entry of Ca\(^{2+}\) into the ER, which leads to the depletion of Ca\(^{2+}\) in the ER and increases cytosolic Ca\(^{2+}\) concentrations (65).
Tunicamycin (Figure 5) is an antibiotic that blocks the synthesis of N-linked glycoproteins in the ER (67). This blockage causes proteins to misfold and aggregate within the ER, which causes ER stress.

With the knowledge that neurodegenerative disease is caused by a variety of factors, and ghrelin has an effect on many characterized apoptotic and inflammatory pathways, we wanted to see if ghrelin had an effect on the less-distinguished ER stress pathway, which is activated in many neurodegenerative diseases. In this study we induced ER stress and looked at ghrelin’s effects on apoptotic, or cell death, indicators.
Caspase -3 and -7, and identified candidate genes regulated by ghrelin and ER stress that may be important in neuroprotection by this hormone.
Methods

Cell Culture

The human neuroblastoma cell line, SH-SY5Y, was used for this study (American Type Cell Culture, Manassas, VA). Parental SH-SY5Y cells were generously provided by Dr. Jeremy Chambers (Scripps Florida) and were maintained in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12: Invitrogen Life Technologies, Carlsbad, California) and supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals, Lawrenceville, Georgia), 1% Penicillin-Streptomycin-Amphotericin B (Pen-Strep; Lonza Walkersville Inc., Walkersville, MD) in a 5% CO₂ humidified incubator at 37° Celsius. These cells were used to create a stable cell line, SY5YGHGR, expressing the human GHSR1a by Dr. Heidi E. Walsh (Scripps Florida). Briefly, SH-SY5Y cells were electroporated with a CMV-driven expression vector containing a hemagglutinin (HA) tag at the N-terminus of the human GHSR1a coding region and a G418 resistance gene. These cells were maintained in the medium above supplemented with 400 µg/mL G418 (Invitrogen) to select for GHSR-expressing cells. A mixed pool of GHSR-expressing cells were stained with a fluorescently-tagged anti-HA antibody (Invitrogen) to confirm expression of the receptor; functionality of the receptor in this stable mixed pool was confirmed by measuring IP-1 accumulation with a commercially available kit (Cisbio, Bedford, MA). Receptor expression was also confirmed by RT-PCR using primers specific for the human GHSR1a. The stable mixed pool was used for all experiments labeled.
SY5YGHSR

To passage, cells were washed with phosphate buffered saline (PBS) and then incubated with 2mL of Accumax (Innovative Cell Technologies, Inc., San Diego, CA) at 37º Celsius for 3-5 minutes. Medium was added to neutralize the reaction and cells were counted and either used for experiments or divided to seed more cell cultures.

Hormones and Inhibitors

Human Ghrelin was obtained from Phoenix Pharmaceuticals and used at 100nM unless otherwise indicated.

Transient Transfections and Reporters

Cells were transfected using FuGENE® HD (Roche Applied Science, Indianapolis, IN) for the SY5YGHSR cells and a total of 0.5 µg total plasmid per well for 16-18 hours. The Luciferase reporter plasmid contained 3 copies of the monomeric NBRE binding site, which was generously provided by Demetrios Vassilates (Academy of Athens).

qRT-PCR

Total RNA was isolated using the Qiagen RNeasy kit; 1µg RNA was utilized for reverse transcription (Superscript III; Invitrogen). mRNA-specific TaqMan probes (Applied Biosystems, Foster City, CA) for NR4A2, HMOX1, NTRK2, BCL2, PARKIN and β-actin were used for real-time PCR. Fold change in mRNA levels were calculated using the ΔΔ Ct using β-actin to normalize. Quantification of mRNA is done through TaqMan probes that have covalently attached fluorophores. As primers are extended in
the polymerase chain reaction (PCR), the fluorophore is released and the fluorescence is detected within the cycler. The amount of fluorescence is directly proportional with the amount of DNA template present in the PCR.

**Labeled Ghrelin Binding and Competition**

Female C57Bl/6J mice were euthanized by CO$_2$ in accordance with Scripps Florida IACUC guidelines, and brains were immediately dissected, frozen in liquid nitrogen, and stored at -80º Celsius until cryosectioning. Mounted brain slices (40µm) were incubated with 100nM Cy5-labeled ghrelin for 20 minutes at 4º Celsius (±10µM unlabeled ghrelin for competition), washed with PBS, and fixed with 10% formalin. For immunostaining, slices were permeabilized with 0.1% Triton-X in TBS for 20 minutes, blocked in 5% BSA, and incubated overnight at 4º Celsius with 1º antibody (anti-GFAP, 1:250, Millipore, Billerica, MA; anti-tyrosine hydroxylase, 1:100, Cell Signaling). Slices were then incubated in 2º antibody (goat anti-rabbit Alexa 488, Invitrogen) for 1 hour, washed with Tris-Buffered Saline-Tween (TBST), and coverslips mounted. Images were obtained using the Olympus Fluoview FV1000 Inverted Confocal Microscope.

**Immunohistochemistry (IHC)**

Experimental animals were anesthetized and euthanized by 80mg/kg ketamine and 16mg/kg citidine in accordance with Scripps Florida IACUC guidelines and perfused transcardially with an aCSF solution (150mM NaCL, 10mM HEPES (free acid), 3mM KCL, 2mM CaCl, MgCl, and d-glucose) followed by 4% paraformaldehyde. Brain samples were post-fixed with 4% paraformaldehyde overnight and equilibrated in 30%
sucrose. Coronal sections of 55µm were prepared with a Leica SM 2010 R freezing sliding microtome. Brain sections were preserved using 0.1% Sodium Azide (Sigma) and kept at 4º Celsius until immunohistochemistry was done. Mounted Brain slides were washed in TBS, permeabilized with 0.1% Triton-X in TBS for 1 hour, blocked by 5% BSA in TBS for 1 hour and 1º antibodies were applied (see concentrations below) and incubated overnight at 4º Celsius. Slices were then washed with TBST and were incubated in 2º antibody for 1 hour at Room Temperature (RT), washed with TBST, and coverslips mounted and sealed using VectaShield mounting media with 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Images were obtained using the Olympus Fluoview FV1000 Inverted Confocal Microscope.

**Inhibitors**

1. Thapsigargin: blocks intracellular Ca+2 flux, 100nM (Sigma)

2. Tunicamycin: blocks synthesis of all N-linked glycoprotein (Sigma).

**Antibodies and Stains**

Anti GFAP 1:250 (Millipore)

Alexa Fluor 488 goat anti-rabbit 1:250 (Invitrogen)

Hoechst 33342, trihydrochloride, trihydrate (Invitrogen)

MitoTracker® Green FM (Invitrogen)

Cy5-Labeled ghrelin (Cisbio, Bedford, MA)
Caspase and Cellular Viability Assays

Caspase 3/7/9 levels were all detected through commercially available Caspase-Glo 3/7 and Caspase- Glo 9 kits and manufacturer’s instructions were followed (Promega, Madison, WI). Cellular viability was measured with Adenosine Triphosphate (ATP) concentrations using a CellTiter- Glo Luminescent Cell Viability Assay (Promega). All luminescent assays were measured using an Envision 2104 Multilabel Reader (PerkinElmer, Covina, California).

Statistical Analysis

Data were analyzed with GraphPad Prism 5. Statistical differences were calculated with a student t-test (unpaired). $p$-values less than 0.05 (*) were considered significant. Errors are Standard Error of the Mean.
Results

Active Caspase-3 and Caspase-7 levels

Previous studies have found that ghrelin decreased active caspase-3 levels in the presence of oxygen-glucose deprivation, but it was unclear whether ghrelin also blocked caspase-3 activation during ER stress (44). We wanted to examine ghrelin’s effect on the apoptotic pathways when activated by ER stress using SY5YGHSR neuroblastoma cells. It is known that prolonged ER stress leads to apoptosis, and to confirm, we used confocal microscopy to visually demonstrate this connection. In order to induce ER stress, SY5YGHSR cells were treated with either Thapsigargin or Tunicamycin for 5 hours, and then labeled with the nuclear stain Hoechst 33342, and the mitochondrial stain MitoTracker® Green FM (Figure 6).

![Figure 6: Live SY5YGHSR imaging during ER stress. A) Normal neuronal cells treated with vehicle (DMSO). Nuclear staining is shown in blue; mitochondrial staining is shown in green. B) Single neuronal cell that treated with 10µg/ml Tunicamycin for 5 hours to induce ER stress.](image-url)
The cells treated with Tunicamycin showed morphological changes, including blebbing, or the formation of plasma membrane bulges, which is a signature characteristic of apoptosis (69-71). To further support the case that ER stress leads to apoptosis we looked at cellular viability of cells with prolonged treatments of Tunicamycin. To determine cellular viability we used the Cell Titer-Glo Luminescent assay, which detects Adenosine Triphosphate (ATP) in cell lysate (Figure 7).

**Figure 7**: Normalized ATP levels in cells treated with 2µg/ml and 5µg/ml Tunicamycin overnight

This experiment supports the idea that ER stress leads to apoptosis, and results in cell death. A 2µg/ml treatment of Tunicamycin produced a 24.12% decrease in cellular viability while a 5µg/ml treatment with Tunicamycin lead to a 23.91% decrease in cellular viability. With the knowledge that ER stress leads to apoptosis, and ER stress is found in neurodegenerative diseases, we wanted to explore ghrelin’s effects on the apoptosis pathway during ER stress. To observe changes in apoptosis we measured active
caspase-3 and caspase-7 as markers in order to determine whether apoptosis was occurring. We first treated cells with 100nM ghrelin and measured caspase-3/7 activity after 1 hour, 5 hours, and overnight treatments (Figure 8, 9, 10).

**Figure 8:** Active caspase-3 and caspase-7 levels in control and experimental cells treated for 1 hour

**Figure 9:** Active caspase-3 and caspase-7 levels in control and experimental cells treated for 5 hours
Figure 10: Active caspase-3 and caspase-7 levels in control and experimental cells treated overnight

At all time points, ghrelin lowered active caspase-3/7 baseline levels. There was a 31% decrease of caspase-3/7 at 1 hour, 38% decrease at 5 hours, and 71% decrease for the overnight treatment when compared to each control condition. Since ghrelin decreased baseline caspase-3/7 levels in neurons, we wanted to determine whether ghrelin had the same effect in the presence of ER stress.

Treating cells with 5µg/ml and 10µg/ml of Tunicamycin for a 5-hour period resulted in an increase in active caspase-3/7 (Figure 11, 12). When 100nM of ghrelin was added in combination with Tunicamycin, a significant decrease in active caspase-3/7 levels was observed.
Figure 11: Active caspase-3 and caspase-7 levels with 5\(\mu\)g/ml ± 100nM Ghrelin treated cells for 5 hours

Figure 12: Active caspase-3 and caspase-7 levels with 10\(\mu\)g Tunicamycin ±100nM Ghrelin treated cells for 5 hours
5µg/ml and 10µg/ml Tunicamycin treatment increased active caspase-3/7 levels by 45% and 107% compared to the vehicle. In the presence of ghrelin, we saw a 20% and 65% decrease in active caspase-3/7 levels compared to the Tunicamycin treatments. As ghrelin had a larger effect on cells treated with the higher concentration of Tunicamycin all further experiments were conducted using 10µg/ml of Tunicamycin.

Changes in Gene Expression in Response to Ghrelin and ER Stress

Next, we wanted to look at the expression of the major anti-apoptotic regulator BCL2, which encodes for the protein Bcl-2 that combats Bax in the fight to initiate apoptosis (Figure 3). Our hypothesis was that ghrelin would increase gene expression of BCL2 and would decrease caspase-3/7 activity, as others have reported that ghrelin increases Bcl-2 protein levels (44). However, using qRT-PCR, there was no change in BCL2 gene expression in ghrelin-treated SY5YGHHR cells (Figure 13).

![BCL2](image)

**Figure 13:** Change in BCL2 gene expression with 100nM ghrelin treatment in SY5YGHHR cells.
The next gene of interest was PARKIN, which encodes a protein that is involved in protein degradation and is known to protect neurons against neurodegeneration (72). We hypothesized that ghrelin would increase PARKIN gene expression and would be a possible pathway that ghrelin affects in neuroprotection. After treating neurons with 100nM of ghrelin for various times, PARKIN levels did not show any increases, and were actually significantly decreased at several time points (Figure 14).

![PARKIN gene expression with 100nM ghrelin treatment in SY5YGHRS cells](image)

**Figure 14**: Change in PARKIN gene expression with 100nM ghrelin treatment in SY5YGHRS cells

Another gene that we were interested in was Neurotrophic tyrosine kinase receptor, type 2 (NTRK2). NTRK2 encodes a receptor that binds the very important protein brain-derived neurotrophic factor (BDNF), which is responsible for neuronal maintenance and neurogenesis. We wanted to see if ghrelin would have a positive effect on this pathway, and help BDNF in its pro-survival signaling. With treatment of 100nM
ghrelin we see an increase in NTRK2 gene expression (Figure 15). We saw significant increases in NTRK2 levels at 30 minutes of ghrelin (1.4 fold), 2 hours (2.1 fold), 3 hours (1.9 fold), and 5 hours (1.5 fold) (Figure 15).

**Figure 15:** Change in NTRK2 gene expression with 100nM ghrelin treatment in SY5YGHSR cells

Since NTRK2 gene expression increased with ghrelin alone, we wanted to determine whether ER stress and ghrelin together could also regulate this gene (Figure 16). Tunicamycin (1.9 fold) and Thapsigargin (3.7 fold) increased gene expression of
NTRK2, and when these treatments were combined with 100nM of ghrelin (3.7 fold) (8.6 fold), there was a significant increase in NTRK2 gene expression after 5 hours of treatment (Figure 16).

![NTRK2 5 hour treatment chart]

**Figure 16:** Change in NTRK2 gene expression with 100nM ghrelin treatment in combination with 100nM Thapsigargin or 10\(\mu\)g/ml Tunicamycin

The next candidate gene was nuclear receptor subfamily 4, group A, member 2 (NR4A2). NR4A2 is an important transcription factor that helps dopamine producing neurons (dopaminergic neurons) differentiate. The dopaminergic neurons are directly affected in Parkinson’s disease, and NR4A2 has possible protective effects. We wanted to see if ghrelin would have an effect on NR4A2 gene expression and possibly interact with these neuroprotective pathways. With the treatment of 100nM ghrelin we see an increase
in NR4A2 gene expression over time with a peak at 1 hour. NR4A2 gene expression was significantly increased by ghrelin, with expression peaking at 1 hour and returning to baseline by 24 hours (Figure 17A).

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 17:** Change in NR4A2 gene expression and NBRE-luciferase reporter activity. A.) Change in NR4A2 gene expression with 100nM ghrelin treatment B.) Change in NBRE-luciferase reporter activity with 100nM ghrelin treatment

The treatment with ghrelin showed a 11 fold increase in NR4A2 gene expression at 30 minutes, 43 fold increase at 1 hour, 21 fold increase at 2 hours, 8.6 fold increase at 8 hours, 4.9 fold increase at 5 hours, and 5.0 fold increase at 8 hours. To further support that ghrelin increases NR4A2, we looked at NR4A2 activity in SY5YGHSR cells when they were treated with 100nM ghrelin (Figure 17B). NR4A2 activity was determined after we transfected SY5YGHSR cells with a luciferase reporter that would be activated when gene was functional and resulted in luminescence. The experiment resulted in a 2.1 fold increase in luciferase activity at 4 hours, 3.5 fold increase at 6 hours, and a 2.5 fold
increase at 8 hours. Seeing that there were large increases in both gene expression and NR4A family transcriptional activity, we then induced ER stress with Tunicamycin and measured the gene expression levels at 5 hours (Figure 18).

**Figure 18:** Change in NR4A2 gene expression with 100nM ghrelin treatment in combination with 10µg/ml Tunicamycin

Tunicamycin treatment alone increased NR4A2 gene expression 7.9 fold. The combination of Tunicamycin and 100nM ghrelin resulted in a 14 fold increase in gene expression. We then used Thapsigargin to induce ER stress and performed the same experiment (Figure 19).
Figure 19: Change in NR4A2 gene expression with 100nM ghrelin treatment in combination with 100nM Thapsigargin

When inducing ER stress with 100nM Thapsigargin, we saw a 116 fold increase in NR4A2 gene expression. With a combination of both Thapsigargin and 100nM ghrelin we saw a very large 436 fold increase in gene expression.

Expression of HMOX1, a gene involved in heme degradation and oxidative stress protection was also regulated by ghrelin in SY5YGHR cells (Figure 20). Treatment with ghrelin for 5 hours resulted in a 1.3 fold increase in HMOX1 gene expression. We then wanted to see the changes in gene expression under ER stress conditions (Figure 21).
**Figure 20:** Change in *HMOX1* gene expression with 100nM ghrelin treatment for 5 hours

**Figure 21:** Change in *HMOX1* gene expression with 100nM ghrelin treatment in combination with 100nM Thapsigargin or 10µg/ml Tunicamycin

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The treatment of 10µg/ml Tunicamycin resulted in a 107 fold increase in HMOX1 gene expression, approaching statistical significance at p= 0.07. Treatment with 100nM Thapsigargin lead to a 53 fold increase in gene expression; however, this increase was not statistically significant. There was a trend for ghrelin treatment to augment in induction of HMOX1 during Tunicamycin-induced ER stress (200.13 fold increase versus vehicle).

**Immunohistochemistry**

In order to see if GHS-R1a was expressed on astrocytes we used ghrelin conjugated with Cy5, since antibodies for GHS-R1a are unreliable. Using the ghrelin conjugate, we could see binding of ghrelin to the receptor. When mouse brain slices were incubated with labeled ghrelin and subsequently immunostained for an astrocytic marker glial fibrillary acidic protein (GFAP), we observed ghrelin bound to the GHS-R1a receptor and a colocalization of ghrelin binding and astrocytes in the mouse brain (Figure 23; C and D). To confirm the specificity of labeled ghrelin binding, we also conducted a competition experiment. Brain slices were incubated with 100nM of the Cy5-labeled ghrelin and 10µM of unlabeled ghrelin. Since unlabeled ghrelin was in excess (1000 fold), the binding of Cy5-labeled ghrelin was inhibited and no significant Cy5 signal could be detected (Figure 23; A and B).
Figure 22: Astrocyte and ghrelin staining. 40µm mouse brain slice stained with GFAP (green), DAPI (blue), and labeled ghrelin (red). A.) Brain slice with labeled ghrelin competition and GFAP at 20x magnification. B.) Brain slice with labeled ghrelin competition, GFAP, and DAPI at 60x magnification. C.) Brain slice with labeled ghrelin, GFAP, and DAPI at 20x magnification. D.) Brain slice with labeled ghrelin, GFAP, and DAPI at 100x magnification
Discussion

Ghrelin lowers active Caspase-3 and Caspase-7 in SH-SY5Y cells during ER stress.

Caspases 3 and 7 are central to the apoptotic pathway, and their activation by proteolytic cleavage initiates apoptosis. Studies have previously shown that ghrelin lowers active caspase-3 activity in neurons during oxygen-glucose deprivation (OGD) (44). The lowering of these important apoptotic factors result in a decrease in cell death, and is beneficial for the neuronal tissue. Knowing that ghrelin decreases active caspase-3 in a hypoxic environment, we wanted to determine whether ghrelin would have the same effect on neuronal cells in the presence of ER stress. ER stress is caused by the aggregation of misfolded proteins and if unresolved, will lead to the activation of apoptosis. We found ER stress to be of great interest since it has been distinguished as a common characteristic of many neurodegenerative diseases (57). Using active caspase-3/7 levels as a marker for apoptosis, we showed that ghrelin lowers active caspase-3/7 activity during ER stress. The data suggest that ghrelin has a protective effect on neurons and helps them survive when misfolded proteins accumulate in the ER. These results show that the neuroprotective effects of ghrelin are not contained within OGD models, but are expanded to include ER stress, which could lead to a protective affect against neurodegenerative diseases.

Ghrelin has no effect on gene expression of BCL2.

Since we saw a decrease in the activity of caspase-3/7 we knew that the apoptotic pathway was being inhibited with the treatment of ghrelin. The initiation of apoptosis is
dependent on the competition of Bcl-2 and Bax; if Bcl-2 levels outweigh Bax levels, apoptosis will be inhibited. Thus, we hypothesized that ghrelin was increasing the gene expression of BCL2, which would lead to the attenuation of the pro-apoptotic pathway. Furthermore, it has been shown in the OGD model that ghrelin increases the protein concentration of Bcl-2, which led to the inhibition of apoptosis in that system (57). Our results indicated that BCL2 gene expression was not changed with the treatment of ghrelin (Figure 13). The opposite result of our study with prior studies can be explained by showing that we were looking at gene expression and the previous studies looked at protein expression. This suggests that ghrelin might be involved in the translational activity within the neuron and could have an effect on prolonging mRNA degradation, which would result in higher protein expression without the change in gene expression. Further studies that we would like to conduct include looking at both Bcl-2 and Bax protein concentrations, along with BAX gene expression. The results from these experiments could further indicate how ghrelin is protecting cells against apoptosis.

**Ghrelin has no effect on gene expression of PARKIN.**

Next, we studied the regulation of other genes that have been shown to have neuroprotective effects. We wanted to see if ghrelin had any effect on these genes, as this could further explain how ghrelin was decreasing inhibiting apoptosis via the capsase-3/7 pathway. PARKIN is a very important gene that encodes a ubiquitin-protein ligase, and is responsible for degrading proteins that are known to cause neurodegenerative disease (73). It has also been recently shown that mutation or knockdown of PARKIN leads to a buildup in proteins that are directly linked to familial Parkinson’s disease, and an increase
in PARKIN gene expression has been shown to have neuroprotective effects (73, 74). However, ghrelin treatment did not increase PARKIN gene expression, and actually significantly decreased its mRNA levels at several time points in SY5YGHSR cells (Figure 14). Although it is unclear what a ghrelin-induced decrease in PARKIN gene expression means functionally in this system, we do still see a decrease in caspase-3/7 levels and know that ghrelin is rescuing cells from apoptosis. However, from this data we have learned that it is unlikely that PARKIN protein is involved in the neuroprotective pathway of ghrelin.

**Ghrelin significantly upregulates NTRK2 gene expression during ER stress.**

Next we looked at another candidate gene that has been known to have neuroprotective effects. The NTRK2 gene encodes the neurotrophic tyrosine kinase receptor type 2, an important receptor in neurons. This is the receptor that binds brain-derived neurotrophic factor (BDNF), which is known to protect neurons and help new neurons form (75). Furthermore, BDNF has been shown to have a possible role in protection of Alzheimer’s disease (76). With ghrelin treatment, we observed a significant increase in NTRK2 gene expression in SY5YGHSR cells (Figure 15). During Tunicamycin or Thapsigargin-induced ER stress, ghrelin significantly enhanced NTRK2 expression (Figure 16). The increase of NTRK2 gene expression could be a way that the cell increases BDNF signaling efficiency, which could result in pro-survival messages to the cell that would rescue the cell from apoptosis. When we induce ER stress the cell could be increasing the receptor in order to save itself from cell death. The addition of ghrelin to ER stress conditions results in a greater increase in NTRK2 gene expression,
potentially amplifying the cell’s sensitivity to pro-survival signals. Further studies, such as measuring the activation of signaling proteins downstream of NTRK2, must be to determine if ghrelin promotes survival against ER stress through this mechanism.

**Ghrelin significantly upregulates NR4A2 gene expression during ER stress.**

The next candidate gene was NR4A2, which encodes for the transcription factor nuclear receptor subfamily 4, group A, member 2. NR4A2 is an important protein involved in the maintenance and differentiation of dopaminergic neurons found in the midbrain, which is the area affected by Parkinson’s disease (77). NR4A2 is also an important protein involved in anti-inflammatory pathways in the brain (78). Furthermore, NR4A2 induces tyrosine hydroxylase (TH) gene expression, which is essential for dopamine production, and mutations in the NR4A2 gene have been linked to Parkinson’s disease, depression, rheumatoid arthritis, and schizophrenia (78, 79). When we treated SY5YGHNR cells with ghrelin we saw a significant increase in NR4A2 gene expression (Figure 18). To confirm that the increase in NR4A2 gene expression we observed also reflected an increase in functional NR4A2 transcription factor, we transiently transfected SY5YGHNR cells with a luciferase reporter gene driven by 3 copies of the DNA-binding element for NR4A transcription factors (NBRE-luc). Ghrelin treatment significantly increased activity of this reporter gene, indicating that NR4A proteins (NR4A1, NR4A2, and NR4A3) are functional after ghrelin treatment. While this assay does not discriminate between the 3 NR4A family members, NR4A2 is the most highly regulated of these three genes by ghrelin (H. Walsh, unpublished observation).
We next wanted to see what effect ER stress would have on NR4A2 gene expression and saw that Tunicamycin or Thapsigargin also upregulated NR4A2 gene expression. These data suggest that the cell is trying to survive in the presence of ER stress and will upregulate NR4A2 gene expression. When we did combination treatments we saw a further upregulation in NR4A2 gene expression. It has been shown that NR4A2 is involved in neuroprotection through the CREB-dependent neuroprotective pathways (80), and our data may indicate that ghrelin is further helping the cell in its survival mechanism. Further studies will need to confirm activity of NR4A2 protein in the presence of ER stress. Knockdown of NR4A2 expression using RNA interference will determine whether the induction of NR4A2 gene expression confers protection to SY5YGHSR cells undergoing ER stress.

**Ghrelin upregulates HMOX1 gene expression during ER stress.**

The last gene analyzed was HMOX1, which encodes heme oxygenase-1. HMOX1 is an enzyme that is essential in heme catabolism, which is a protein that can become toxic if not degraded in the cells (81). HMOX1 is essential in heme degradation by breaking the molecule into biliverdin. HMOX1 has also been shown to be protective against oxidative stress by interacting with various antioxidant pathways including Nrf2-ARE during induced oxidative stress, and has been linked to neuroprotective pathways (82-85). When we treated neurons with ghrelin we saw a significant upregulation in HMOX1 gene expression (Figure 20) indicating that the neuroprotective effects of ghrelin may be mediated by the heme oxygenase-1 pathway.
With the induction of ER stress, we saw upregulation of HMOX1 activity with both Thapsigargin and Tunicamycin treatments (Figure 21). When we treated with ghrelin during ER stress, we saw trends that would indicate that ghrelin further upregulates HMOX1 gene expression. There are no significant differences since we had such a low sample number, and further studies will need to be done in order to validate ghrelin’s upregulation of HMOX1 in the presence of ER stress.

**GHS-R1a may be expressed in astrocytes in mouse brain.**

Astrocytes are glial cells found in the brain and are very important in neuronal maintenance and are part of the brain’s local immune system (86, 87), and studies have shown that astrocytes are linked to genes we have shown to be upregulated by ghrelin. For instance, NR4A2 has been shown to decrease inflammatory cytokine production in astrocytes to protect dopaminergic neurons from inflammation-induced death. Other studies have shown that NR4A2 work to block astrocytic and microglial pathways that induce inflammation within the brain and result in a NR4A2 neuroprotective effect (78, 88). Recently, upregulation of HMOX1 in astrocytes has also been shown to protect dopaminergic neurons through HMOX1’s byproduct bilirubin (89). It has also been shown that HMOX1 is involved in anti-inflammatory pathways involving astrocytes by being upregulated in the presence of pro-inflammatory molecules (90). Since ghrelin is neuroprotective in vivo and regulates both NR4A2 and HMOX1 in vitro, we wanted to determine whether GHS-R1a was expressed by astrocytes. Since there is no reliable antibody for the receptor, we used a Cy5-labeled ghrelin conjugate and incubated the labeled ghrelin with mouse brain slices, which would bind to the GHSR-1a if present and
produce a visible signal. Interestingly, we observed colocalization of labeled ghrelin and the astrocyte marker protein glial fibrillary acidic protein (GFAP) in mouse brain (Figure 22). This colocalization suggests that GHSR-1a could be expressed on the membrane of astrocytes and thus ghrelin may regulate neuroprotective pathways through the brain’s internal immune system. Ghrelin has already been shown to have an influence on the global immune system. It has been shown that ghrelin is expressed in T cells, which are the major players in immune response (91). Furthermore, ghrelin decreases proinflammatory cytokines expression in human T cells, which suggests that ghrelin may regulate immune cell activation and inflammation (91, 92). Studies must be done to further demonstrate the expression of GHS-R1a on astrocytes. The interactions between astrocytes and ghrelin must be further analyzed, but if the data that we have found is validated through other methods including gene and protein expression, we may have uncovered an important novel interaction that ghrelin has within the brain which would help us better understand how ghrelin is mediating neuroprotection.
Works Cited


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