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Infection with Chlamydia Pneumoniae in Neuronal Cells Alters the Expression of Genes Involved in Apoptosis and Autophagy Pathways

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INFECTION WITH *CHLAMYDIA PNEUMONIAE* IN NEURONAL CELLS ALTERS THE EXPRESSION OF GENES INVOLVED IN APOPTOSIS AND AUTOPHAGY PATHWAYS

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Abstract

Dysfunctions in cellular mechanisms such as apoptosis and autophagy have been implicated in the neurodegeneration associated with Alzheimer's disease (AD). Autophagy in AD pathogenesis has been linked to the endosomal-lysosomal system, which has been shown to play a role in amyloid processing. Studies have suggested that apoptosis may contribute to the neuronal cell loss observed in AD; however, there is no evidence of the apoptotic process leading to terminal completion. Aβ1-42 has been shown to induce apoptosis in neurons and may be an initiating factor in AD. Our previous studies demonstrated that neurons infected with *C. pneumoniae* are resistant to apoptosis, and that Aβ1-42 was increased by the infection. Additionally, studies have demonstrated the interactions of several pathogens on the autophagic pathway. The focus of the current studies was to determine if there is a relationship between the molecular mechanisms interconnecting autophagy and apoptosis following *C. pneumoniae* infection in neuronal cells that could lead to the pathologies observed in AD. SKNMC neuronal cells obtained from ATCC were infected with the AR39 strain of *C. pneumoniae* at an MOI=1 for 24hrs to 72hrs and were analyzed using Real-time PCR arrays from SABiosciences specific for autophagy and apoptosis markers. At 24hrs, neuronal cells infected with *C. pneumoniae* resulted in down-regulation of apoptosis and autophagy gene regulation. Neuronal cells infected at 72hrs with *C. pneumoniae* up-regulated the expression of several genes associated with autophagy and apoptosis. For example, BECN1 and ATG4C, both prominent genes in the autophagy pathway were 3.66 to 25.77 fold down-regulated at 24hrs and increased 1.53 to 1.73 fold 72hrs post-infection,

respectively. Similarly, apoptosis gene expression such as that for BAK1 was increased from -1.15 fold at 24hrs to 2.09 fold at 72hrs post-infection. Our data suggest that *C. pneumoniae* exerts a control over changes in gene regulation affecting the apoptotic and autophagic processes in neuronal cells. As both autophagic and apoptosis dysfunction have been observed in AD, the impairment of these normal cellular processes by a pathogen such as *C. pneumoniae* may contribute to the neuropathology seen in AD.

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Chapter 1: Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to a loss of memory and cognitive function in the elderly. Changes in behavior and personality are also predominant symptoms in AD patients (1). Age is one of the major risk factors for AD, and individuals over 80 years of age are at the greatest risk for developing AD (2). AD is classified as either sporadic, also referred to as late onset, or the familial form. Sporadic AD usually occurs in individuals 65 years of age or older, and affects ~50% of Americans who are 85 years and older (1), whereas familial AD occurs in 2% to 5% of AD cases. Familial AD can manifest during a person's thirties, forties, or fifties (1), and is associated with genetic mutations, such as mutations in the genes encoding the amyloid precursor protein (APP), presenilin-1 and presenilin-2 proteins (3).

A confirmed diagnosis of AD cannot be made until a histological examination of the patient's brain is performed at autopsy. At the macroscopic level, it is very difficult to distinguish the brain of an AD patient from a non-AD patient of the same age because many of the gross anatomical structures are similar. For example, an aging brain and an AD brain will present with enlarged sulci and atrophied gyri. Although, if the hippocampus has undergone much atrophy, and there is an enlargement of the temporal horn, these abnormalities together may be indicative of AD. However, microscopic examination of the brain is used to determine if AD is the proper diagnosis (4).

1.2 Hallmark Pathologies associated with Alzheimer's disease

Two hallmarks of AD are the presence of neurofibrillary tangles and neuritic senile plaques (2), which are initially found in the entorhinal cortex and hippocampus of AD brains (1). Neurofibrillary tangles (NFTs) are thick, abnormal fibrils that form in the cell bodies of neurons. Paired helical filaments are the major protein structure that collectively forms the NFTs (5). The paired helical structures consist mainly of tau, a protein that is associated with microtubules (6). The tau protein is hyperphosphorylated in the paired helical filaments and is formed into neurofibrillary tangles (NFTs) (7). Tranglutaminase has been implicated in the formation of NFTs by enzymatically crosslinking tau into the insoluble filamentous NFT structures (8).

Neuritic senile plaques are found in the extracellular space that surrounds neurons (9). The β-amyloid (Aβ) peptide is the main constituent of neuritic senile plaques (10). The proteolytic cleavage of amyloid precursor protein (APP) can result in the formation of Aβ (11) and can be cleaved through the non-amyloidogenic pathway or the amyloidogenic pathway (12). In the non-amyloidogenic pathway, APP is cleaved at the plasma membrane by α-secretase to form soluble APP (APPsα) and a C-terminal fragment that is 83 amino acids long (C83). At the endosome membrane, C83 is cleaved by γ secretase to form p3, a derivative of the beta-amyloid peptide, and the intracellular amyloid precursor protein cytoplasmic/C-terminal domain (12). In the amyloidogenic pathway, APP is taken into the cell via endocytosis. The cleavage of APP by β-secretase at the endosome membrane results in the formation of the secreted ectodomain, APPsβ, and a C-terminal fragment that is 99 amino acids long, C99 (12). C99 is then cleaved by

γ-secretase to form $A\beta_{40}$ and $A\beta_{42}$ (12, 13). $A\beta_{40}$ is more commonly produced than $A\beta_{42}$, however, $A\beta_{42}$ is more toxic than $A\beta_{40}$ (10, 11). The cleavage products, APPsβ, $A\beta_{40}$, and $A\beta_{42}$ are secreted out of the cell to form amyloid plaques (12). The predominant form of Aβ found in the neuritic senile plaques is $A\beta_{42}$ (10).

Current research indicates that sequential cleavage of the APP by ε-secretase and then γ-secretase also results in the formation of Aβ. The cleavage of APP by ε-secretase results in $A\beta_{49}$ and $A\beta_{48}$, which when cleaved by γ-secretase forms the products $A\beta_{46}$ and $A\beta_{43}$ from $A\beta_{49}$, and $A\beta_{45}$ from $A\beta_{48}$. Cleavage of $A\beta_{46}$ and $A\beta_{43}$ by γ-secretase then results in $A\beta_{40}$, and $A\beta_{42}$ is formed when $A\beta_{45}$ is cleaved by γ-secretase (11).

Recently, mitochondrial dysfunction has been described as a feature characteristic of neurons and astrocytes of postmortem AD brains. Studies have found that mitochondria in brains from AD patients experienced oxidative stress (14). Some studies also suggest that the mitochondrial membranes in the neurons of AD patients contained a buildup of Aβ oligomers. These investigations suggest that essential proteins cannot pass through the mitochondrial membranes containing Aβ oligomers, which would result in dysfunction of the electron transport chain, an increase in reactive oxygen species, and changes in the structure of the mitochondria (14, 15). A reduction in the number of mitochondria that function appropriately in neurons, especially in the terminal regions of neurons, may contribute to poor transmission between neurons that could result in a decrease in cognitive function (14, 15).

1.3 Apoptosis

Mitochondrial dysfunction can lead to apoptosis, a form of cell death. Apoptosis occurs at regulated levels in the cells of the body; however, if apoptosis becomes unregulated and decreases or increases too much within specific cells, such as neuronal cells, apoptosis could be associated with diseases, such as AD. A cell that is undergoing apoptosis exhibits specific characteristics including condensation of chromatin, shrinkage of the cell, bleb formation, and nicking of the DNA. Caspases are active upon initiation of apoptosis in a cell and is a characteristic that is associated with apoptosis (16).

Apoptosis can be initiated by various stimuli acting on different receptors (17). The more common pathways include the death receptor pathway and the mitochondrial pathway. The activation of a death receptor by a death ligand stimulates caspase-8, which then cleaves the Bid protein or caspase-3; an effector caspase that induces apoptosis. The activation of the Bid protein leads to the activation of the Bax and Bak proteins, which will result in cytochrome c being released from the mitochondria. The release of cytochrome c leads to the activation of caspase-9, which also will cleave caspase-3 to result in the stimulation of apoptosis (17).

1.4 Forms of Autophagy

In some instances, cells may activate the autophagy pathway instead of, or before, initiating apoptosis (18). An increase in the number of autophagic vacuoles (AVs) has been identified in neurons from AD brains implicating autophagy as a pathological process in AD (19). There are three different forms of autophagy, all of which result in

the lysosomal degradation of proteins and organelles. In microautophagy, the cytoplasmic components of a cell are engulfed by lysosomal membranes. Chaperone-mediated autophagy occurs when proteins with a KFERQ-like peptide motif are imported directly across the lysosomal membrane. Macroautophagy is a process where the cytoplasm and its constituents within a cell are engulfed by a double-membrane structure referred to as an autophagosome (18). The formation of an autophagosome is coordinated by complexes consisting of autophagy gene (ATG)-related proteins (20). Autophagosome formation occurs within the synapses and neuritic processes of neurons. Because lysosomes are most commonly found in the neuronal cell bodies, in order for an autophagosome to fuse with a lysosome, retrograde transport of the autophagosome to the cell body of the neuron must be efficient (21). The lysosomal hydrolases within the lysosome will then degrade the contents of the autophagosome (18, 20, 22). The proteins and other substances that are broken down by lysosomal hydrolases can be used for other cell processes or energy requirements (18, 19, 22). Macroautophagy is the form of autophagy most commonly studied (18).

1.5 Autophagy and the Endosomal-Lysosomal System

Autophagy is associated with the endosomal-lysosomal system since autophagosomes will fuse with late endosomes or lysosomes (22). Studies have found that the activity of the lysosomal system is enhanced in patients with AD by observing an increase in the production of hydrolases present in lysosomes (23). The lysosomal system is also related to the endosomal pathway since early endosomes that are formed

will develop into late endosomes, which exchange their content or fuse with lysosomes (21). Neurons from AD brains have been found to exhibit enlarged early endosomes. This is significant in AD because early endosomes take in proteins, such as apolipoprotein E and APP, and it has been determined that Aβ is formed in early endosomes (23). The formation of Aβ has also been observed within AV's. The accumulation of AV's in the AD brain, possibly because of impaired AV clearance and autophagy induction, may therefore promote the accumulation of Aβ (21).

1.6 Autophagic Vacuole Pathology in Alzheimer's disease

The autophagic vacuole pathology observed in AD has been compared to that seen in autophagic dysfunction during various stages of the autophagy pathway in primary rat cortical neurons. While the induction of autophagy alone by rapamycin did not result in AV pathology similar to that observed in AD brains, AD-like AV pathology was seen to result from impaired clearance of AV's after treatment with vinblastine or leupeptin. Vinblastine treatment was shown to slow the fusion of autophagosomes with lysosomes; and leupeptin, a protease inhibitor, inhibits the degradation of proteins in lysosomes (24). Clearance of autophagosomes is also impaired when their retrograde transport to the neuronal cell body for fusion with lysosomes has been impaired as has been observed in AD (21). The impaired clearance of AV's, alone or in combination with induction of autophagy, can produce an AV pathology which is similar to that observed in the AD brain (24).

1.7 Autophagy Signaling Pathways

The process of autophagy is regulated by two main signaling pathways; each acting on the mammalian target of rapamycin (mTOR) kinase, a serine/threonine kinase (25), which is an important regulator of autophagy (20). There is a decrease in autophagy when stored amino acids and growth factors, such as insulin, are increased. In one signaling pathway, AMP-activated protein kinase (AMPK) is inhibited when there is an increase in the concentration of intracellular amino acids. This results in the TSC1/TSC2 complex not being active, since AMPK activates the TSC1/TSC2 complex. The activity of the GTP-binding protein Rheb will therefore be increased when the TSC1/TSC2 complex is inactive, which results in the activation of mTOR kinase to inhibit autophagy (20).

Growth factors signal the second pathway that regulates autophagy by activation of the Class I phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, which also leads to a decrease in autophagy by activating mTOR kinase (20). The activation of the Class I PI3K-Akt/PKB pathway via growth factors inhibits the tuberous sclerosis complex (TSC1/TSC2) by stimulating phosphorylation, which results in the Rheb protein being active. The active form of Rheb will activate mTOR kinase, which results in a decrease in autophagy (18, 20).

There are specific kinases, such as mTOR kinase, that function through phosphorylation of protein complexes involved in the induction or inhibition of autophagy. For instance, when mTOR kinase is active it phosphorylates the ULK complex in order to inhibit the complex from inducing autophagy. The ULK complex

consists of the ULK1 and ULK2 kinases. The inhibition of mTOR kinase allows the ULK complex to be active, which induces autophagy (18).

The next complex to be activated in the autophagy pathway, the Beclin1-Class III PI3K complex, which requires the interaction and activation of the proteins UVRAG, Atg14, and Ambra1. The active form of the Beclin1-Class III PI3K complex consists of PI3-P-enriched membrane domains, which interact with proteins such as LC3, Atg9, and the Atg5-12-16 complex. These proteins are needed for the formation of an autophagosome. Another protein that is involved in autophagosome formation is Bif-1, which interacts with UVRAG and the Beclin1-Class III PI3K complex. Bif-1 has been found to result in the curving of membranes, and may be associated with the development of the autophagosome membrane (18).

Proteins such as Bcl2, Beclin 1, p53, Atg5, Atg4d, and the DAP kinases have been found to interact with proteins in both the autophagy and apoptosis pathways (18, 25). For instance, the anti-apoptotic Bcl2 protein can bind to the BH3 domain of the Beclin 1 protein and prevent Beclin 1 from inducing autophagy (18). The p53 protein is known to induce apoptosis; however, under physiological stress p53 has also been found to increase the rate of expression of the DRAM gene, and can inhibit mTOR through AMPK to induce autophagy (25). Both Atg5 and Atg4d are proteins involved in autophagy that can be cleaved, which results in these proteins inducing apoptosis after moving to the mitochondria (18, 25). The DAP kinases are known to regulate apoptosis through phosphorylation and DAP kinase will also phosphorylate Beclin 1, which decreases Beclin 1's binding to Bcl-2 so that autophagy may be induced (18).

1.8 Neuroinflammation

Another characteristic seen in the brains of AD patients is neuroinflammation (26). Neuroinflammation has also been associated with other neurodegenerative disorders. When glial cells in the brain are continually activated, neuroinflammation occurs. The activation of glial cells results in the release of proinflammatory mediators, which are thought to be involved in the pathology of AD. The Aβ peptide contained in the senile plaques that are associated with AD have been found to attract microglial cells, which are a type of activated glial cell. The increased expression of tryptophan metabolites has also been observed in AD, which results in the presence of IgA antibodies in the serum to the tryptophan metabolites. The increased expression of tryptophan metabolites occurs when the indoleamine 2,3-dioxygenase (IDO) pathway is activated (27).

1.9 Chlamydia pneumoniae

Infection with *C. pneumoniae* and other microorganisms, such as herpes simplex virus type 1, can promote pathology that is associated with sporadic AD (28). Amyloid plaques, similar to those found in AD brains, were identified in the brains of BALB/c mice following infection with *C. pneumoniae* (29). Inflammation also occurred in these brains after infection with *Chlamydia pneumoniae*. Studies have shown that *C. pneumoniae* DNA is found in AD brain tissue samples taken postmortem, than from brain tissue samples of people who did not present with AD pathology (28). *C. pneumoniae* is thought to enter the host's brain through the olfactory tract and/or the blood brain barrier.

Using PCR and RT-PCR, it was determined that *C. pneumoniae* was present in the olfactory bulbs and cells that are located near blood vessels of post-mortem AD patients (30).

C. pneumoniae is an obligate intracellular bacterium that infects the respiratory tract. *C. pneumoniae* was originally referred to as TWAR, which is a combination of the two codes, TW-83 and AR-39, used to identify the first *C. pneumoniae* isolates (31). *C. pneumoniae* has a biphasic life cycle that begins with an elementary body attaching to the host cell and entering the host cell through endocytosis. The elementary body is the infectious form of *C. pneumoniae* that is metabolically inactive. In the endosome, the elementary bodies can form an inclusion and become reticulate bodies, which are not infectious and are metabolically active. The reticulate bodies replicate and are then converted back to elementary bodies. After approximately 48hrs to 72hrs the elementary bodies are released by lysis of the host cell (32).

1.10 Evidence of Effects of Chlamydia pneumoniae on Apoptosis and Autophagy

The effects of infection with different species of *Chlamydia* on the processes of apoptosis in monocytes and neuronal cells and autophagy in cervical carcinoma HeLa cells have been studied (33, 34, 35). In monocytes it was found that *Chlamydia pneumoniae* infection leads to the activation of NF-κB, which results in the inhibition of apoptosis (33). Similarly, when neuronal cells were infected with *C. pneumoniae* and then treated with staurosporine to induce apoptosis, apoptosis was inhibited (34). In contrast, autophagy was induced following infection of cervical carcinoma HeLa cells

with *Chlamydia trachomatis* for 24hrs, which was thought to be due to low nutrient levels in the infected cells (35).

1.11 Proposed Studies

Mitochondrial dysfunction and the initiation of the apoptotic process have been observed in the pathology in AD brains (16). However, there is a lack of evidence demonstrating that apoptosis has advanced to completion within the neuronal cells in AD. Since *C. pneumoniae* has been implicated as a trigger in the development of AD pathology and has been shown to inhibit apoptosis (34), these studies focused on investigating an alternate pathway that may be initiated by *C. pneumoniae* in the infected cell. Autophagy may be the alternative pathway that is up-regulated in an attempt to maintain a state of homeostasis within the neuronal cell.

There's a complex relationship between autophagy and apoptosis in a cell's adaptation in avoiding cellular death (25). Naturally evolving cellular mechanisms for survival may override the self-destructive apoptosis pathway by alternatively inducing autophagy. The induction of the autophagy pathway is a survival response for the cell in a highly regulated cellular process by gene and protein regulation to eliminate damaged organelles and proteins that would be detrimental to the cell. Interestingly, intracellular pathogens, such as different species of *Chlamydia*, have been shown to alter the apoptosis pathway and the autophagy pathway to ensure survival of the host cell (33, 34, 35). Therefore, our experiments were designed to see what effects *Chlamydia pneumoniae* has on autophagy and apoptosis in neuronal cells, which have both been implicated in AD.

We hypothesize that infection with *C. pneumoniae* in neuronal cells may exert pressure inducing autophagy as seen in AD. In our experimental paradigm, changes in gene expression following an infection with *C. pneumoniae* were measured using RT^2-PCR arrays specific for autophagy and apoptosis. SKNMC neuronal cells were infected with *Chlamydia pneumoniae* at an MOI = 1 for 24hrs and 72hrs in this study. The experiments were performed in triplicate for both uninfected and infected samples that were run on the arrays for each time point, and uninfected samples were run on the RT^2-PCR arrays as controls.

Chapter 2: Materials and Methods

2.1 Tissue Culture

SKNMC neuronal cells (Cat. # HTB–10 - ATCC) were cultured in growth media (GM) containing Minimum Essential Medium Eagle (Cat. # 30-2003 - ATCC) and 10% FBS. Cells were maintained at 37°C in 5% CO_2. For propagation, neuronal cells were grown in T25 corning flasks (Cat. # - 430639) in a total volume of 8mL GM. For the infection assays, cells were plated at 5.0 x 10^4 for the 72hr infection, and 2.0 x 10^5 to 5.0 x 10^5 for the 24hr infection in 6 well culture plates (Cell Star, Cat. # 657 160). Cells were grown to a confluency of approximately 50% at which time the cells exhibited a neuronal morphology.

2.2 Establishment of Cell Counts

Cell counts were established for an infection with the AR39 strain of *Chlamydia pneumoniae* (*C. pneumoniae*) (Cat. # 53592 – ATCC) by trypsinizing (Cat. # 15400-054 - Gibco) one of the wells in the 6 well plate, and neutralizing the trypsin with GM. The cells were pelleted by centrifuging at 1,000X g in the Spectrafuge (Cat. # C2400-P -24D Labnet) for 5 minutes at room temperature (RT). The supernatant was removed and the cells are resuspended in 1ml of GM. 10µl of cells were mixed with 10µl of trypan blue (Cat # T1082, 837949- Invitrogen) and were counted using the Countess automated cell counter (Cat # C10281- Invitrogen).

2.3 Infection of neuronal cells with Chlamydia pneumoniae

All infections of neuronal cells were performed at an MOI (multiplicity of infection) = 1 with *C. pneumoniae* in 6 well plates once the cells were grown to a 50% confluency displaying a neuronal morphology. At the start of each infection, GM was removed from each well, and 1ml of fresh GM was added to each well. At this point, cells that were to remain uninfected were incubated at 37°C in 5% CO_2 for 24 or 72 hrs. A total volume of 1mL, comprised of GM and the appropriate concentration of *C. pneumoniae*, was added to the wells for the infection assay. The *C. pneumoniae* was thawed, sonicated for 1 minute, and vortexed for 1 minute, and then added directly to the wells. The infected 6 well plate was then inserted into a biohazard bag and centrifuged at RT for 30 minutes at 1,000X g in the Sorvall Legend RT. Following centrifugation, the cells were incubated at 37°C in 5% CO_2 for 24 or 72 hrs. For the 72hr time point, 1ml of GM was added to the infected and the uninfected wells 24hrs after initiation of the infection with *C. pneumoniae*. Following incubation, GM was removed, and the cells were rinsed in Hank's Balanced Salt Solution (HBSS) (Cat. # 30-2213 – ATCC) prior to processing for RNA extraction and immunocytochemistry.

2.4 Apoptosis Induction with Staurosporine

For apoptosis induction at 24 and 72 hrs post-infection in both uninfected and infected cells in the 6 well plates, the GM was removed. A 1mL solution of 1µM staurosporine (Cat. # 078K4057, S6942 - Sigma) in GM was added, and then incubated for 3hrs at 37°C in 5% CO_2. Following incubation, GM containing the staurosporine was

removed, and the cells were rinsed in MEM (ATCC) prior to processing for RNA extraction and immunocytochemistry.

2.5 Screening for Chlamydia pneumoniae infection by Immunocytochemistry

Cells grown in 6 well plates from 8.0 x 10^5 to 1.5 x 10^6 were detached with 700µl of trypsin and neutralized with 700µl of GM to remove the cells from the wells. The cells were then pelleted at 1000X g in the spectrafuge for 5 minutes at RT. The supernatant was discarded and the pellet was resuspended in 1mL of GM. 500µl of GM containing the cells were transferred into EZ Single cytofunnels (Cat. # A78710020 Shandon- ThermoScientific) containing adhesive glass slides (Cat. # 7255/90 - Starfrost), and were cytospun with the Thermoshandon Cytospin3 (Cat # 74000222 Issue 8) for 4 minutes at 32X g. The glass slide was removed and the cells were fixed in cytofix/cytoperm (BD Biosciences #554722) for 30 minutes. The cells were rinsed twice with PBS, and then blocked in 500µl of 1X perm/wash (Cat. # 51-2091KZ – 554723 – BD Biosciences) for 30 minutes. The cells were rinsed 2X with PBS (Cat. # P3813 – 10PAK – 109K8210), then once with molecular grade water. The cells were incubated at 37° C for 1 hour in FITC conjugated *C. pneumoniae* antibody 61C75 (Fitzgerald) diluted in PBS at 1:100. The cells were rinsed with PBS twice, and then rinsed 5X with molecular grade water. The cells were coverslipped using mounting media containing DAPI (Cat. # 17985-51 - EMS Fluoro Gel II).

Slides were viewed on a Nikon 90I and captured with Kodak element program. The neuronal cells that were infected with *C. pneumoniae* were counted to determine the

percentage of cells infected at each time point. For the 24hr time point, cells were counted on slides from three different 24hr infections. There were three to four slides for each 24hr infection. One field of cells from each slide was counted. In each field for the 24hr infections, there were 26 to 98 total cells. For the 72hr time point, cells were counted on slides from two different 72hr infections. There were two slides for each 72hr infection. Similar to the 24hr time point, one field of cells from each slide was counted for the 72hr time point. In each field for the 72hr infections, there were 37 to 99 total cells.

2.6 Processing for RNA Extraction

Media was removed from infected or uninfected cells grown in the 6 well plates. A set of cells were then treated with staurosporine for 3 hours. The cells were rinsed with either HBSS or MEM and then incubated with 700µl of trypsin for 3 minutes followed by the addition of 700µl of GM. The solution of cells from each well was centrifuged in microcentrifuge tubes for 5 minutes at 1,000X g in the spectrafuge. Following centrifugation, the supernatants were removed and the cells were resuspended in HBSS. Cell counts were obtained from each well to ensure a cell count of at least 4.0×10^5 for the RNA extraction. Following cell counts, the cells were pelleted at 1,000X g in the spectrafuge for 5 minutes. The supernatants were discarded and the pellets were chilled on ice and then stored at -80°C prior to RNA extraction.

2.7 RNA Extraction

All RNA extraction and cDNA protocols were performed in the PCR hood (AirClean 600PCR Workstation – ISC BioExpress). All reagents and equipment were cleaned with an RNaseZap wipe (Cat. # AM9786) prior to being put in the PCR hood. All centrifugations were performed in the spectrafuge. 350µl of Buffer RLT (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen) was added to the pelleted cells that had been previously frozen at -80°C, and then vortexed. The lysate was pipetted into a QIAshredder spin column placed in a 2 mL collection tube (QIAshredder (50) Cat. # 79654 – Qiagen) and centrifuged in the spectrafuge for 2 minutes at 16,300X g. The QIAshredder spin column was discarded and 350µl of 70% ethanol was mixed with the homogenized lysate in the collection tube. 700µl of the homogenized lysate was transferred to an RNeasy spin column placed in a 2ml collection tube (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen). The sample was then centrifuged in the spectrafuge for 15 seconds at 11,200X g. The flow through was discarded and 350µl of Buffer RW1 (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen) was added to the RNeasy spin column to wash the spin column membrane. The sample was centrifuged for 15 seconds at 11,200X g in the spectrafuge, and the flow through was discarded.

An optional DNase digestion was performed to eliminate genomic DNA contamination. 70µl of Buffer RDD and 10µl of DNase I stock solution (RNase-Free DNase Set (50) Cat. #79254 – Qiagen) (RNase-Free DNase Set (50) Cat. # 79254 – Qiagen) was added into a 0.5ml PCR tube (Cat. # AM12275 – Ambion). This DNase I incubation mix was centrifuged for 15 seconds at 11,200X g in the spectrafuge. 80µl of

the DNase I incubation mix was then added directly to the RNeasy spin column membrane, and incubated at RT for 15 minutes. 350µl of Buffer RW1 was added to the RNeasy spin column, and the sample was centrifuged for 15 seconds at 11,200X g. The flow through was discarded and 500µl of Buffer RPE (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen) was added to the RNeasy spin column to wash the spin column membrane. The sample was centrifuged in the spectrafuge for 15 seconds at 11,200X g. 500µl of Buffer RPE was added to the RNeasy spin column to wash the spin column membrane, and the sample was centrifuged for 2 minutes at 11,200X g. Following centrifugation, the RNeasy spin column was carefully placed in a new 1.5ml collection tube (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen), and 40µl of RNase-free water (RNeasy Mini Kit (50) Cat. # 74104 – Qiagen) was added directly to the spin column membrane. The sample was centrifuged for 1 minute at 11,200X g to elute the RNA.

2.8 Determination of RNA Yield

5µl of the RNA sample was mixed with 55µl of 10mM Tris pH 8, or 10µl of RNA sample was mixed with 990µl of 10mM Tris pH 8, and these samples were centrifuged for 5 – 10 seconds at 11,200X g. The sample was then added to a quartz cuvette, either 60µl (Cat. # 14–385–928F – Fisher Scientific) or 1mL cuvette (Cat. # 14–385–914A) for determination of the RNA concentration. The samples were read with a spectrophotometer (Nicolet evolution 100 – ThermoScientific). The spectrophotometer was blanked with 10mM Tris pH 8, and the absorbancies were recorded at 260nm and 280nm. The absorbancy at 260nm was used to determine the RNA yield with the

equation 44ug/ml x A_{260} x dilution factor; the dilution factor was 12 for the 60µl cuvette or 100 for the 1mL cuvette. The absorbancies at 260nm and 280nm were used to calculate the ratio, A_{260}/A_{280}, which indicated if the sample had any protein contamination. A number between approximately 1.8 and 2.0 indicated that there was no protein contamination of the RNA sample.

2.9 First Strand cDNA Synthesis

The RNA stock that had been stored at -80°C was used to prepare the cDNA for an RT^2-PCR human autophagy PCR array (RT^2 Profiler™ PCR Array Human Autophagy – SABiosciences PAHS – 084A). Approximately 1µg of RNA was used to generate the cDNA for each array.

For the first strand cDNA synthesis, the determined amount of RNA sample and 2µl of 5X gDNA Elimination buffer (RT^2 First Strand Kit – cDNA Synthesis – Cat. # C03/330401 - SABiosciences) was added to a sterile 0.2ml PCR tube (Cat. # AM12225 - Ambion). The nuclease-free water (Cat. # AM9930 – Ambion) was added to the PCR tube bringing the total volume of genomic DNA elimination mixture to 10µl. The genomic DNA elimination mixture was centrifuged for 10 seconds at 6,000X g. The mixture was incubated in the PCR machine (Techne TC-3000G) at 42°C for 5 minutes. Following incubation, the genomic DNA elimination mixture was chilled on ice for at least one minute. For preparation of the RT cocktail (RT^2 First Strand Kit – cDNA Synthesis – Cat. # C03/330401 - SABiosciences), 4µl of 5X RT Buffer 3 (BC3), 1µl of Primer & External Control Mix (P2), 2µl of RE3 (RT Enzyme Mix 3), and 3µl of

nuclease free water were added to a sterile 0.2mL PCR tube. The 10µl of RT cocktail was added to the genomic DNA elimination mixture. The reaction was incubated in the PCR machine at 42°C for 15 minutes and the reaction was stopped by heating at 95°C for 5 minutes. Following heating, 91µl of nuclease free water was added to the cDNA and held on ice until the array was prepared or the cDNA was stored at -20°C overnight.

2.10 Real-Time PCR Human Autophagy Arrays

The experimental cocktail was prepared by mixing 1,248µl of nuclease free water, 102µl of diluted first strand cDNA synthesis reaction, and 1,350µl of 2X SABiosciences RT^2 qPCR Master Mix (Cat. # PA-012-12 – SABiosciences) into a reservoir (Cat. # B-0820-8 – BioExpress). The RT^2-PCR array was opened in the PCR hood and placed in a PCR isofreezer. An eight-channel pipettor was used to add 25µl of the experimental cocktail to each well of the PCR Array, one column at a time. After addition of the cocktail to the column, the wells were sealed with an 8-cap strip. The RT^2-PCR array was centrifuged in the Sorvall Legend RT centrifuge for 3 minutes at RT and 1,000 rpm. Real-time PCR (RT-PCR) was performed using the Applied Biosystems Prism 7000 Sequence Detection System to measure the PCR amplification from the autophagy array.

2.11 Data Analysis

For the data analysis, the changes in fold regulation of the autophagy and apoptosis gene profiles for the neuronal cells were determined for both time points by using SABiosciences PCR Web-Based Data Analysis. The threshold cycle data from

each RT^2-PCR array run was uploaded into SABiosciences PCR Web-Based Data Analysis website, which would convert the threshold cycle data into the values for the changes in fold regulation. The threshold cycle data obtained from uninfected neuronal cells from each time point was used as the controls for the untreated infected neuronal cells for each time point, respectively. The threshold cycle data obtained from uninfected neuronal cells treated with staurosporine from each time point was used as the controls for the treated infected neuronal cells for each time point, respectively. For further analysis of apoptotic gene expression, infected neuronal cells were induced into apoptosis with 1µM staurosporine, which is a protein kinase C (PKC) inhibitor (36). The changes in autophagy gene expression was also studied to see if infection with *C. pneumoniae* following staurosporine treatment would lead to induction of autophagy, since previous studies have shown that apoptosis is inhibited following *C. pneumoniae* infection (34).

Chapter 3: Results

3.1 Verification of Infection by Immunocytochemistry

For verification of infection with *C. pneumoniae*, the neuronal cells were immunolabeled with FITC conjugated *Chlamydial* antibody 61C75A. The percentage of infected cells was determined from counting profiles of cells containing a positive labeling for *C. pneumoniae* (Figure 1). For the 24hr time point, 50% of the cells were infected, whereas 70% of the cells were infected at the 72hr time point (Figure 2). For each time point that demonstrated satisfactory immunolabeling of *C. pneumoniae*, the RNA was extracted from the uninfected and infected neuronal cells and was converted into cDNA. The uninfected neuronal cells were screened on the RT^2-PCR arrays as controls for each time point.

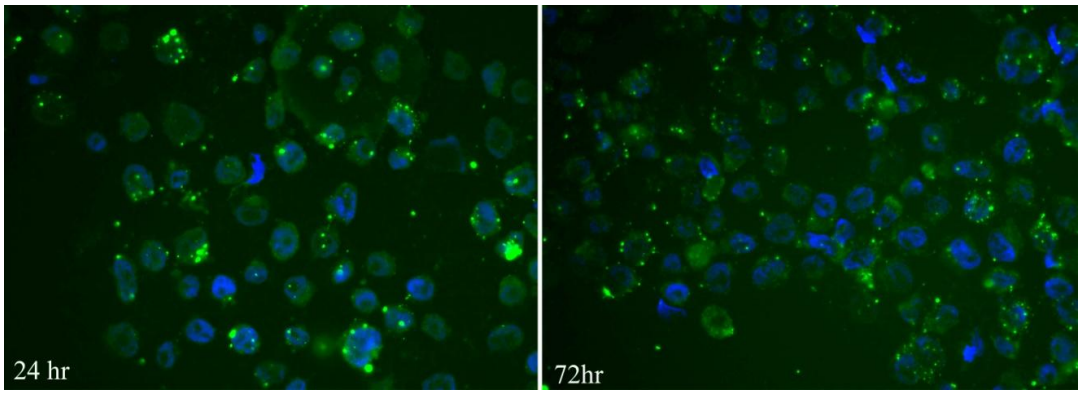

Figure 1. Neuronal cells immunolabeled with FITC conjugated Chlamydial antibody 61C75A following infection with C. pneumoniae for 24hrs and 72hrs. Green represents the C. pneumoniae and blue is the nuclei of the cell.

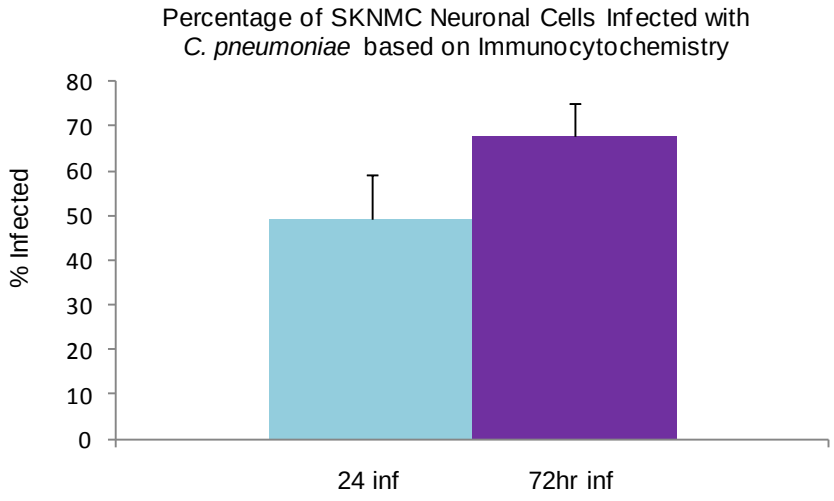

Figure 2. Quantitation of SKNMC neuronal cells that were infected with C. pneumoniae at 24hrs post-infection and 72hrs post-infection based on immunolabeling of cells with FITC conjugated Chlamydial antibody 61C75A.

3.2 Apoptosis Genes

In neuronal cells infected with *C. pneumoniae*, as seen in figure 3 and table 1, a selection of key genes specific for apoptosis showed a downward trend in gene expression at the 24hr time point and an upward trend in gene expression at the 72hr time point. The genes that demonstrate this trend are BAK1, BNIP3, CASP8, and NFKB1. The gene that demonstrated the most pronounced downward trend at 24hrs post-infection was NFKB1 with a 49.505 fold down-regulation. However, the gene expression for NFKB1 was not as suppressed at 72hrs, but remained down-regulated by a factor of 4.057. Interestingly, the NFKB1 gene is typically up-regulated following a bacterial infection in other cell lines, such as monocytes (Dr. Balin & Charlie unpublished). The BAK1 and BNIP3 genes encode proteins that contribute to apoptosis through the

mitochondrial pathway (37). Alternatively, the CASP8 gene functions as an initiator caspase to induce apoptosis in the death receptor pathway. The fold regulation of the BAK1, BNIP3, and CASP8 genes at 24hrs was between -1.0 and -2.2, whereas at 72hrs the range of fold regulation was between -1.1 and 2.1. One of the genes that did not follow the previously described trend was the FAS gene, which encodes a death receptor that is a member of the TNF-superfamily (38). The interaction of the Fas death receptor with proteins such as caspase 8 is involved with the initiation of the apoptotic pathway (38). The fold regulation at 24hrs for the FAS gene was 4.298, and was decreased to 3.295 at 72hrs.

Table 1. Fold regulation of the key genes involved in apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation of Apoptosis Genes		
	24hr inf	72hr inf
BAK1	-1.150	2.096
BNIP3	-1.437	1.722
CASP8	-2.193	-1.130
FAS	4.298	3.295
NFKB1	-49.505	-4.057

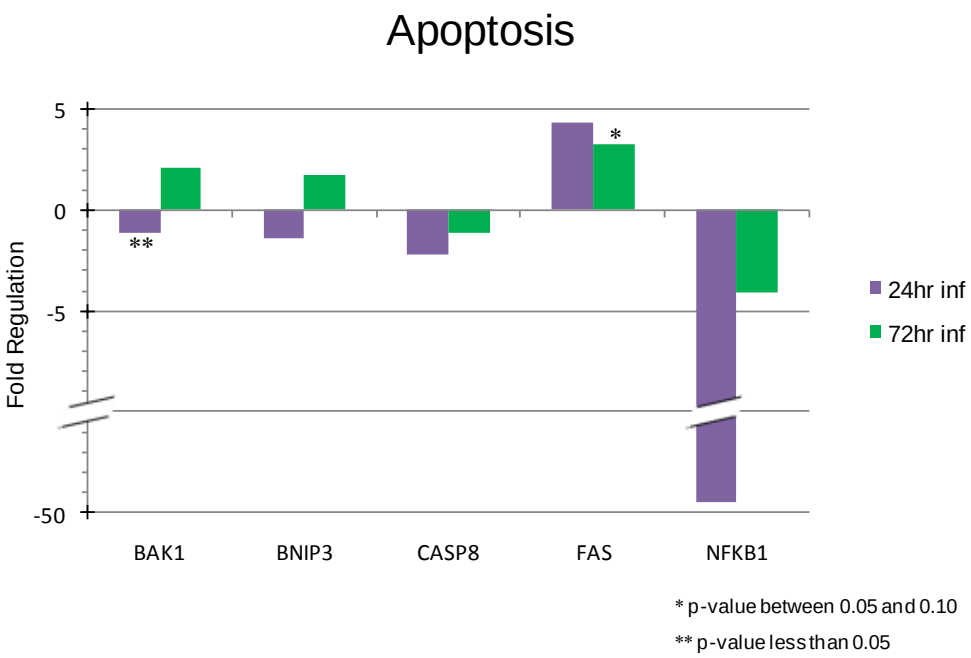

Figure 3. Fold regulation of the key genes involved in apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated with asterisks.

3.3 Apoptosis Genes Following Staurosporine Treatment

In order to differentiate the effects exerted by *C. pneumoniae* in neuronal cells on the pro-apoptotic genes, the neuronal cells were induced into apoptosis with 1µM staurosporine. As seen in figure 4 and table 2, an incubation of the infected neuronal cells with staurosporine resulted initially, at 24hrs, in an up-regulation of the key genes involved in apoptosis, such as BAK1, BNIP3, CASP8, FAS, and NFKB1, followed by a decrease in fold regulation at the 72hr time point. The most prominent change in gene expression occurred from 24hrs to 72hrs with the CASP8, FAS, and BNIP3 genes. At 24hrs, the CASP8 gene increased by 3.595 fold, and then decreased to

-1.096 fold at 72hrs. Similarly, the FAS gene had an 8.877 fold increase in expression at 24hrs, which was suppressed to 1.432 fold at 72hrs. Initially, the fold regulation for the BNIP3 gene was 1.555 at 24hrs, which decreased to -3.917 fold at 72hrs. Interestingly, NFKB1 has a minimal change in gene expression from 24hrs to 72hrs in the presence of staurosporine. A comparison between the infected neuronal cells and the infected neuronal cells treated with staurosporine as seen in figure 4, revealed a reversal in gene expression. The effects exerted by *C. pneumoniae* specifically indicated that *C. pneumoniae* is suppressing the pro-apoptotic genes at 72hrs.

Table 2. Fold regulation values for the key genes involved in apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation - Apoptosis				
	24hr inf	72hr inf	24hr inf +stauro	72hr inf +stauro
BAK1	-1.151	2.096	1.974	1.263
BNIP3	-1.437	1.722	1.555	-3.917
CASP8	-2.193	-1.130	3.595	-1.096
FAS	4.298	3.295	8.877	1.432
NFKB1	-49.505	-4.057	2.548	2.479

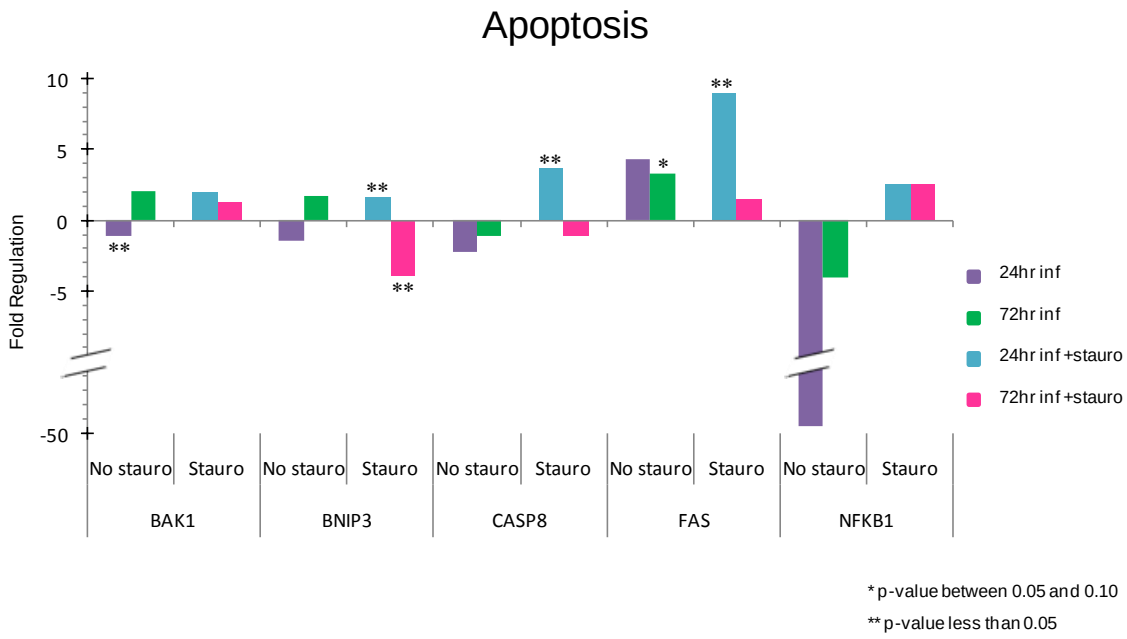

Figure 4. Fold regulation for the key genes involved in apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs and treatment with staurosporine for 3hrs to induce apoptosis. Genes with significant p-values are indicated with asterisks.

3.4 Autophagy Genes

Since there is a commonality in the triggering mechanisms of apoptosis and autophagy, genes specific for autophagy were also screened following infection of neuronal cells with *C. pneumoniae* from 24hrs to 72hrs. Some key genes involved in autophagy include ATG4C, ATG9A, BECN1, and DRAM. The ATG4C, ATG9A and BECN1 genes encode proteins that are pivotal to autophagosome formation (39, 40). The DRAM gene encodes a lysosomal protein that is a damage-regulated autophagy modulator (41). The general trend in the expression of the autophagy genes was a

down-regulation at 24hrs post-infection and an up-regulation after 72hrs post-infection (Figure 5 and Table 3). At 24hrs, the fold regulation for the ATG4C gene was -25.773, which was followed by an increase to 2.738 fold at 72hrs. The fold regulation for the BECN1 gene was -3.662 at 24hrs, which increased to 1.532 fold at 72hrs. The fold regulation for the DRAM gene was -2.680 at 24hrs and 1.575 at 72hrs. There was a minimal change in gene expression for ATG9A.

Table 3. Fold regulation values for the key genes involved in autophagy following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation of Autophagy Genes		
	24hr inf	72hr inf
ATG4C	-25.773	2.738
ATG9A	-1.067	1.637
BECN1	-3.662	1.532
DRAM	-2.680	1.575

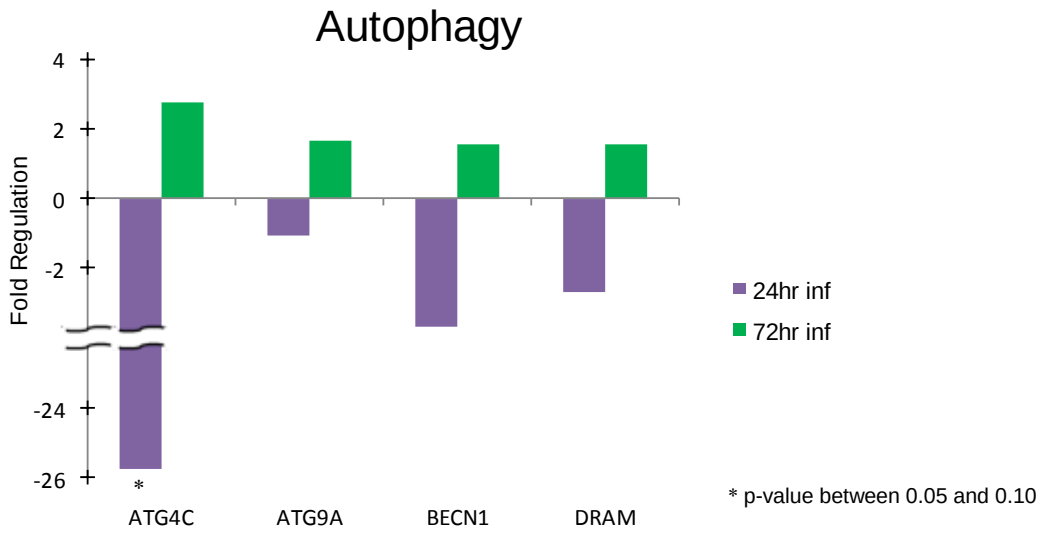

Figure 5. Fold regulation of the key genes involved in autophagy following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated by an asterisk.

3.5 Genes involved in Autophagy Induction by Intracellular Pathogens

Genes that are involved in autophagy induction by intracellular pathogens, such as IFNA2, IFNA4, and IFNG, were analyzed following infection of neuronal cells with *C. pneumoniae* at 24hrs and 72hrs. The IFNA2 and IFNA4 genes encode cytokines that stimulate immune cells (42). The protein encoded by IFNG is a cytokine that activates macrophages (38). At 24hrs, the IFNA4 and IFNG genes were slightly up-regulated, whereas the IFNA2 gene was slightly down-regulated. Each of these genes was up-regulated after 72hrs (Figure 6 and Table 4). The IFNA4 gene had a fold regulation of 1.747 at 24hrs, and was up-regulated to 3.184 fold at 72hrs. There was a fold regulation of -1.176 at 24hrs for the IFNA2 gene, which was up-regulated to 2.522 fold at 72hrs. At

24hrs the IFNG gene had a fold regulation of 1.506 and a fold regulation of 2.852 at 72hrs.

Table 4. Fold regulation values for the genes relevant to autophagy induction by intracellular pathogens following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation – Autophagy Induction by Intracellular Pathogens		
	24hr inf	72hr inf
IFNA2	-1.176	2.522
IFNA4	1.747	3.184
IFNG	1.506	2.852

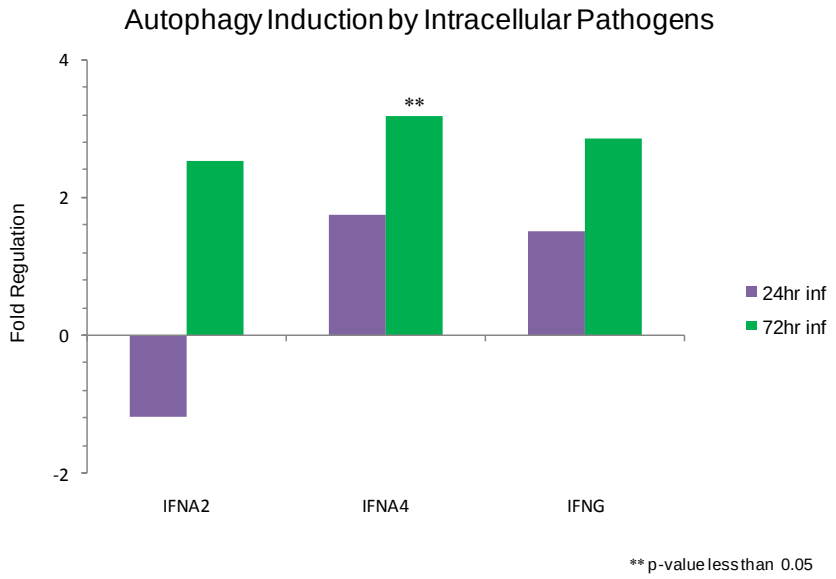

Figure 6. Fold regulation of the genes associated with autophagy induction by intracellular pathogens following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with a significant p-value are indicated with asterisks.

3.6 Autophagy Genes Following Staurosporine Treatment

The neuronal cells were induced into apoptosis with 1µM staurosporine to compare the effects of *C. pneumoniae* and staurosporine in neuronal cells on the key genes involved in autophagy. As seen in figure 7 and table 5, the treatment of infected neuronal cells with staurosporine resulted in minimal up-regulation of the key genes involved in autophagy, which include ATG4C, ATG9A, BECN1, and DRAM, at 24hrs and 72hrs. For all of the key autophagy genes following staurosporine treatment, except for ATG9A, there was a greater level of expression at 24hrs than at 72hrs. For example, with staurosporine the ATG4C gene had a fold regulation of 1.999 at 24hrs and 1.485 at 72hrs. In contrast, the ATG9A gene had a fold regulation of 1.481 at 24hrs with staurosporine and a fold regulation of 2.107 after 72hrs with staurosporine. At 72hrs, the fold regulation of the key autophagy genes following treatment of infected neuronal cells with staurosporine was similar to the fold regulation of the key autophagy genes from the untreated infected neuronal cells.

Table 5. Fold regulation values for the key autophagy genes following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs and treatment with staurosporine for 3hrs to induce apoptosis.

Fold Regulation of Autophagy Genes				
	24hr inf	72hr inf	24hr inf +stauro	72hr inf +stauro
ATG4C	-25.773	2.738	1.999	1.485
ATG9A	-1.067	1.637	1.481	2.107
BECN1	-3.662	1.532	1.767	1.690
DRAM	-2.680	1.575	1.654	1.563

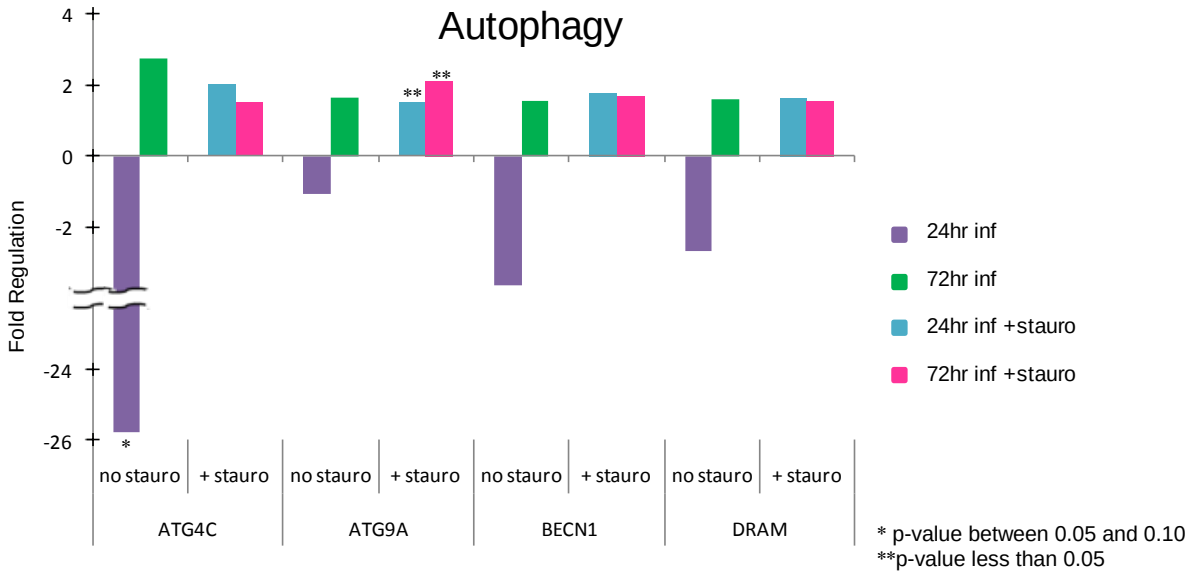

Figure 7. Fold regulation of the key genes involved in autophagy following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs and treatment with staurosporine for 3hrs to induce apoptosis. Genes with significant p-values are indicated with asterisks.

3.7 Autophagy Machinery Components

The RT^2-PCR array includes multiple genes involved in the autophagy process. The autophagy genes are divided into two categories, such as genes for autophagy machinery components and genes involved in the regulation of autophagy. Autophagy machinery components refer to genes associated with autophagic vacuole formation, protein targeting to the membrane/vacuole, protein transport, linking of the autophagosome to the lysosome, and protease activity (43).

A majority of the genes associated with autophagy machinery components were up-regulated in the neuronal cells infected with *C. pneumoniae* for 24hrs and 72hrs, with greater up-regulation after 72hrs (Figure 8 and Table 6). The primary genes involved

with autophagic vacuole formation include AMBRA1, ATG16L1, and MAP1LC3B. The protein encoded by the AMBRA1 gene is associated with autophagy by controlling turnover of proteins (38), and the Ambra1 protein interacts with the Beclin 1-Class III PI3K complex, which is a part of the autophagy signaling pathway (18). The ATG16L1 gene encodes a protein that is part of a large protein complex necessary for autophagy, while the MAP1LC3B gene encodes a protein that is a subunit of the neuronal microtubule-associated MAP1A and MAP1B proteins (39). At 24hrs, the AMBRA1 gene was down-regulated whereas the ATG16L1 and MAP1LC3B genes were up-regulated. The fold regulation for the MAP1LC3B gene was 2.131 at 24hrs and 1.216 at 72hrs. The ATG16L1 gene had a fold regulation of 1.456 at 24hrs, and an increase in fold regulation to 2.336 after 72hrs. In contrast to the general trend observed for the genes implicated in autophagy machinery components, the AMBRA1 gene was down-regulated to 2.112 fold at 24hrs, and was up-regulated to 1.375 fold at 72hrs.

The predominant genes associated with protein targeting to the membrane/vacuole and protease activity are ATG4A and ATG4D (Figure 8 and Table 6). The ATG4A and ATG4D genes encode proteins that are cysteine proteases, which are necessary for autophagy (38). Both genes were up-regulated at the 24hr and 72hr time points, with greater up-regulation after 72hrs. At 24hrs, the ATG4A gene had a fold regulation of 1.465, which was up-regulated to 2.115 fold at 72hrs. The ATG4D gene had a 1.228 fold regulation at 24hrs and a fold regulation of 1.422 at 72hrs.

The ATG4A and ATG4D genes are also involved in protein transport, as are the ATG10, ATG16L1, and RAB24 genes (Figure 8 and Table 6). The ATG10 gene encodes

an E2-like enzyme, which is involved in ubiquitin-like modifications, such as the conjugation of Atg12 and Atg5 for autophagosome formation (38). The protein encoded by the RAB24 gene is a GTPase, which is a Ras-related protein that is implicated in the regulation of intracellular protein trafficking (38). All of these genes were up-regulated at 24hrs and 72hrs following infection with *C. pneumoniae,* except for the ATG10 gene, which was slightly down-regulated at 24hrs. The RAB24 gene had a fold regulation of 2.517 at 24hrs, which decreased to a fold regulation of 1.049 at 72hrs. After 24hrs, the fold regulation for the ATG10 gene was -1.121, which was up-regulated to 5.360 fold at 72hrs.

FAM176A is a gene involved in the linking of the autophagosome to the lysosome (Figure 8 and Table 6). The FAM176A gene is also known as the TMEM166 gene (43), which is a gene that encodes a lysosome and endoplasmic reticulum-associated membrane protein (44). At both time points, the FAM176A gene was up-regulated, with greater up-regulation after 72hrs, following infection of the neuronal cells with *C. pneumoniae*. For the 24hr time point, the FAM176A gene had a fold regulation of 2.561. After 72hrs, the FAM176A gene had a fold regulation of 2.854.

Table 6. Fold regulation values of genes associated with autophagy machinery components following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation-Autophagy Machinery Components		
	24hr inf	72hr inf
AMBRA1	-2.112	1.375
ATG10	-1.121	5.360
ATG16L1	1.456	2.336
ATG4A	1.465	2.115
ATG4D	1.228	1.422
MAP1LC3B	2.131	1.216
RAB24	2.517	1.049
FAM176A	2.561	2.854

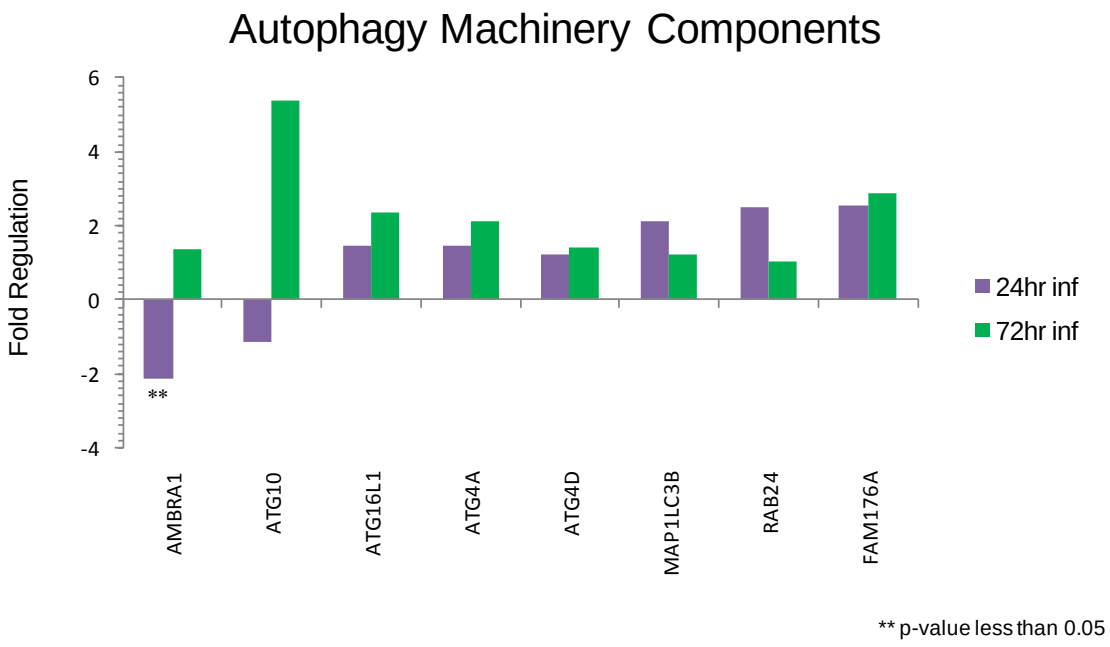

Figure 8. Fold regulation for genes associated with autophagy machinery components following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated with asterisks.

3.8 Regulation of Autophagy

There are autophagy genes that belong to the regulation of autophagy category, which refers to genes that are involved with autophagy in response to other intracellular signals, co-regulators of autophagy and apoptosis, or co-regulators of autophagy and the cell cycle. After infection of the neuronal cells with *C. pneumoniae*, the genes associated with autophagy in response to other intracellular signals, such as EIF4G1, GAA, ULK2, and UVRAG, showed a greater level of expression after 72hrs than at 24hrs (Figure 9 and Table 7). The EIF4G1 gene encodes a protein that is part of a multi-subunit protein complex, which is involved in the initiation phase of protein synthesis (38). The GAA

gene encodes acid alpha-glucosidase, which is necessary for the degradation of glycogen to glucose in lysosomes (38). The protein encoded by the ULK2 gene is important during the activation of autophagy (45). The UVRAG gene encodes a protein that activates the Beclin 1-PI(3)KC3 complex to induce autophagy (38). At 24hrs, the EIF4G1 and GAA genes were up-regulated, and the ULK2 and UVRAG genes were down-regulated. At 72hrs, the EIF4G1, GAA, and UVRAG genes were up-regulated, whereas the ULK2 gene was greatly down-regulated. For example, the GAA gene had a fold regulation of 1.527 at 24hrs, which was up-regulated to 2.946 fold at 72hrs. In contrast, the ULK2 gene had a fold regulation of -1.591 at 24hrs, which was down-regulated to 8.183 fold at 72hrs.

Table 7. Fold regulation values for genes relevant to autophagy in response to intracellular signals following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation - Autophagy in Response to Intracellular Signals		
	24hr inf	72hr inf
ARSA	-3.416	1.733
EIF4G1	2.391	2.725
ESR1	-1.201	2.207
GAA	1.527	2.946
PIK3C3	-1.227	3.088
PIK3R4	2.089	2.726
PRKAA2	2.139	2.023
TMEM74	1.876	2.998
TMEM77	2.263	1.914
ULK2	-1.591	-8.183
UVRAG	-2.440	1.611

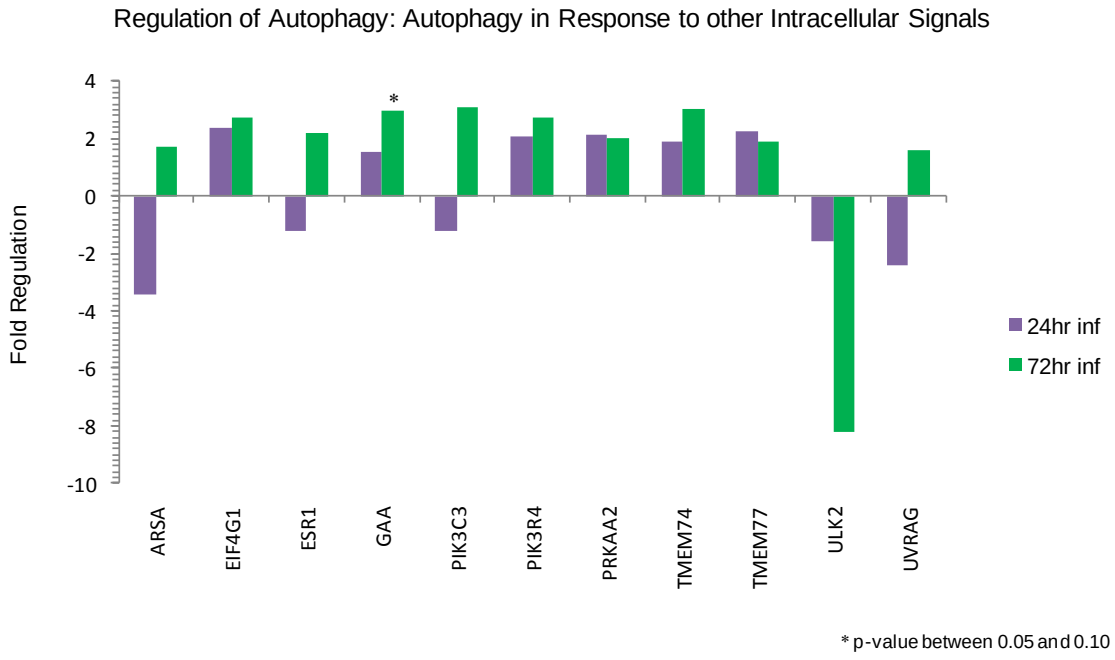

Figure 9. Fold regulation for genes associated with autophagy in response to intracellular signals following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated with an asterisk.

Following infection of neuronal cells with *C. pneumoniae*, as seen in Figure 10 and Table 8, the BAX, CDKN1B, TGFB1, and TP73 genes are co-regulators of autophagy and the cell cycle, which had greater expression at 24hrs than at 72hrs, except for the CDKN1B gene. The expression of the BAX gene is regulated by the tumor suppressor P53. The BAX gene encodes a BCL2 family protein that binds BCL2 to prevent BCL2's anti-apoptotic activity (38). The CDKN1B gene encodes a protein that regulates cell cycle progression by arresting the cell cycle at G1 (38). The protein encoded by the TGFB1 gene is a member of the transforming growth factor family of

cytokines (38). The TP73 gene encodes a member of the p53 family that is part of the apoptosis response in the cell to stress (38). At 24hrs, there was a fold regulation of 2.136 for the BAX gene, 2.807 for the TGFB1 gene, and 3.252 for the TP73 gene. The fold regulation at 72hrs was 1.965 for the BAX gene, 2.580 for the TGFB1 gene, and -5.247 for the TP73 gene. In contrast to the general trend observed for the previous genes, the CDKN1B gene had a fold regulation of -2.858 at 24hrs, which increased to -1.168 fold at 72hrs.

Table 8. Fold regulation values for genes involved in the co-regulation of autophagy and the cell cycle and/or the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation - Co-Regulators		
	24hr inf	72hr inf
AKT1	-3.542	1.286
BAX	2.136	1.965
BCL2	-1.055	1.399
CDKN1B	-2.858	-1.168
CDKN2A	1.889	2.398
CLN3	1.495	2.238
PRKAA1	-1.230	1.314
PTEN	3.890	1.245
SQSTM1	-7.375	2.100
TGFB1	2.807	2.580
TGM2	-1.619	2.162
TNF	4.625	1.640
TP73	3.252	-5.247

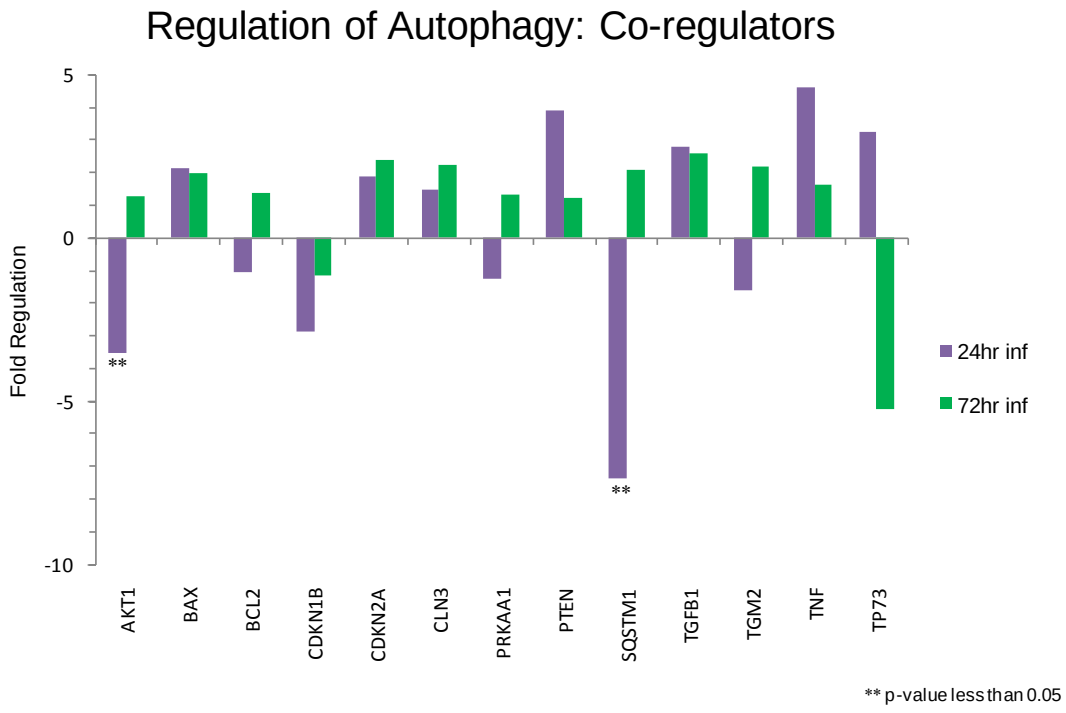

Figure 10. Fold regulation for genes involved in the co-regulation of autophagy and the cell cycle and/or the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated with asterisks.

3.9 Co-regulation of Autophagy and Apoptosis

The key genes that are co-regulators of autophagy and apoptosis include BCL2, PRKAA1, SQSTM1, and TGM2. After infection of the neuronal cells with *C. pneumoniae*, these genes were down-regulated at 24hrs and up-regulated at 72hrs (Figure 11 and Table 9). The BCL2 gene is involved in both apoptosis and autophagy regulation (18). The BCL2 gene encodes an outer mitochondrial membrane protein that suppresses apoptosis (38). The BCL2 protein can also bind with the Beclin 1 protein to

prevent Beclin 1 from inducing autophagy (18). The PRKAA1 gene encodes a catalytic subunit of the 5'-prime-AMP activated protein kinase (AMPK), which can sense the cell's energy levels (38). The SQSTM1 gene encodes a multifunctional protein that binds ubiquitin and regulates the activation of the NFKB signaling pathway (38). The TGM2 gene encodes a transglutaminase enzyme that seems to be involved in apoptosis (38). The BCL2 gene had a fold regulation of -1.055 at 24hrs and a fold regulation of 1.399 at 72hrs. The SQSTM1 gene had a fold regulation of -7.375 at 24hrs, which increased to 2.100 fold at 72hrs.

Table 9. Fold regulation values for the key genes involved in the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs.

Fold Regulation - Co-regulators of Autophagy and Apoptosis		
	24hr inf	72hr inf
BCL2	-1.055	1.399
PRKAA1	-1.230	1.314
SQSTM1	-7.375	2.100
TGM2	-1.619	2.162

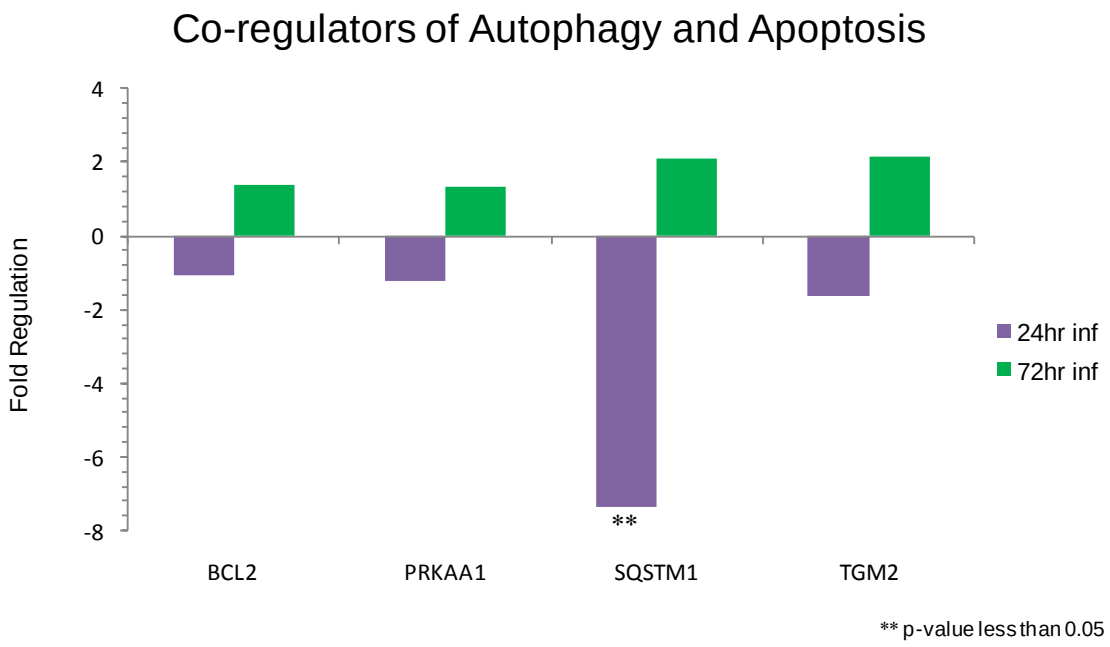

Figure 11. Fold regulation for the key genes involved in the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs. Genes with significant p-values are indicated with asterisks.

3.10 Co-regulation of Autophagy and Apoptosis Following Staurosporine Treatment

In order to differentiate the effects exerted by *C. pneumoniae* in neuronal cells on the key genes involved in the co-regulation of autophagy and apoptosis, the infected neuronal cells were induced into apoptosis with 1µM staurosporine. As seen in figure 12 and table 10, the addition of staurosporine to neuronal cells had varied results for the key genes involved in the co-regulation of autophagy and apoptosis, which include BCL2, PRKAA1, SQSTM1, and TGM2. At 24hrs the BCL2 and PRKAA1 genes were up-regulated, whereas the SQSTM1 and TGM2 genes were down-regulated. At 72hrs, the BCL2, PRKAA1, and SQSTM1 genes were up-regulated, whereas the TGM2 gene was

down-regulated. The BCL2 gene had a fold regulation of 2.046 at 24hrs, which decreased to a 1.208 fold regulation at 72hrs. The TGM2 gene had a fold regulation of -2.449 at 24hrs and -1.133 at 72hrs.

Table 10. Fold regulation values for the key genes involved in the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae and treatment with staurosporine for 3hrs to induce apoptosis.

Fold Regulation - Co-regulators of Autophagy and Apoptosis				
	24hr inf	72hr inf	24hr inf +stauro	72hr inf +stauro
BCL2	-1.055	1.399	2.046	1.208
PRKAA1	-1.230	1.314	3.150	1.580
SQSTM1	-7.375	2.100	-1.874	1.244
TGM2	-1.619	2.162	-2.449	-1.133

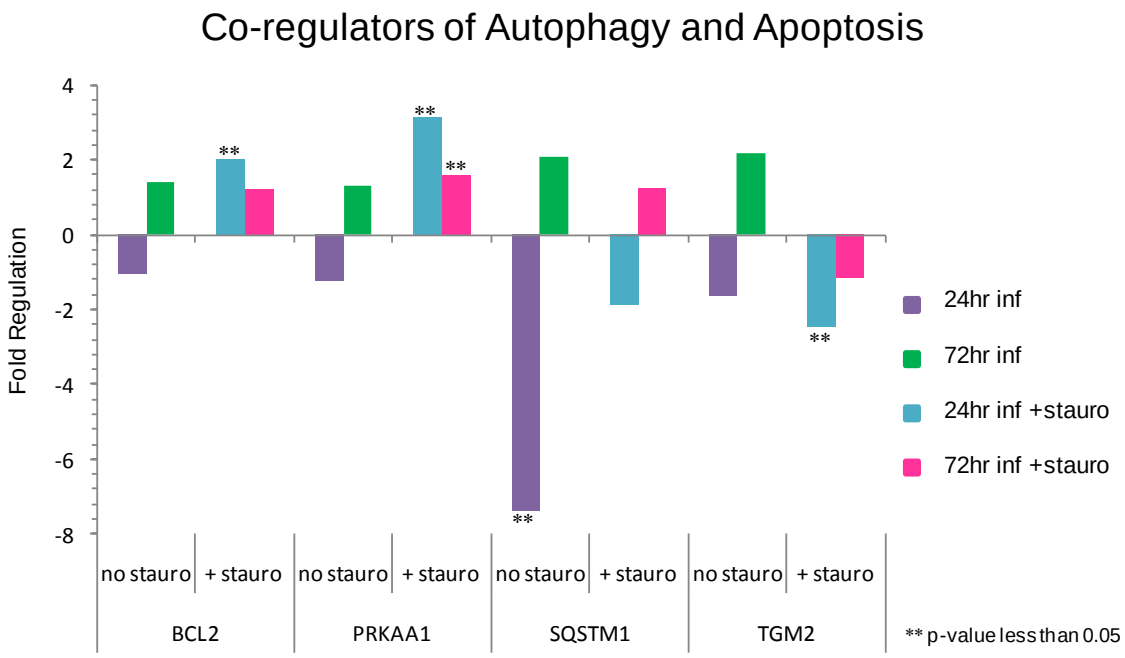

Figure 12. Fold regulation for the key genes involved in the co-regulation of autophagy and apoptosis following infection of neuronal cells with C. pneumoniae for 24hrs and 72hrs and treatment with staurosporine for 3hrs to induce apoptosis. Genes with significant p-values are indicated with asterisks.

3.11 Trends in Autophagy and Apoptosis Data

Following infection of the neuronal cells with *C. pneumoniae*, the general trend demonstrated by the genes involved in both apoptosis and autophagy was a down-regulation of expression at 24hrs followed by an up-regulation at 72hrs. In contrast, following treatment of infected neuronal cells with staurosporine to induce apoptosis, the trends in gene regulation were different for the autophagy and apoptosis genes. After the infected neuronal cells were incubated with staurosporine, the genes

involved in autophagy were up-regulated at both 24hrs and 72hrs; whereas there was an up-regulation of gene expression at 24hrs followed by a down-regulation of gene expression at 72hrs for the genes involved in apoptosis. Uninfected neuronal cells at 24hrs and 72hrs were the controls for the infected neuronal cells. Uninfected neuronal cells incubated with staurosporine at 24hrs and 72hrs were the controls for the infected neuronal cells incubated with staurosporine.

From the 84 genes analyzed on the RT^2-PCR array, we chose to observe 43 of these genes more closely. After infection of the neuronal cells with C. pneumoniae, 7 of the 43 genes had significant p-values. Of these 7 genes, two are involved in apoptosis, which include the FAS and SQSTM1 genes. The other 5 were genes involved in autophagy, including AKT1, AMBRA1, ATG4C, GAA, and IFNA4. The ATG4C, FAS, and GAA genes had p-values approaching significance that were between 0.05 and 0.10. The AKT1, AMBRA1, ATG4C, and SQSTM1 genes had significant p-values at 24hrs post-infection, whereas the FAS, GAA, and IFNA4 genes had significant p-values at 72hrs post-infection.

Following treatment of neuronal cells with staurosporine, an apoptosis inducer, to differentiate the effects of *C. pneumoniae,* 23 of the 43 genes that were observed had significant p-values. The PRKAA2 gene at 24hrs post-infection and the ATG4D and TGFB1 genes at 72hrs had p-values approaching significance that were between 0.05 and 0.10. About half of the 23 genes had significant p-values at both 24hrs post-infection and 72hrs post-infection. A majority of the 23 genes had significant p-values at 24hrs post-

infection. Of the 23 genes, 7 genes are involved in apoptosis and 16 genes are involved in autophagy.

3.12 Summary of Functions of Gene Products

Table 11. Functions of the gene products of each gene that was discussed in the results section.

Gene	Functions of Gene Products
BAK1	Contributes to the mitochondrial pathway of apoptosis (37)
BNIP3	Contributes to the mitochondrial pathway of apoptosis (37)
CASP8	Initiator caspase that induces apoptosis via the death receptor pathway (37)
FAS	Death receptor that induces apoptosis via the death receptor pathway (38)
NFKB1	Inhibits apoptosis and is a nuclear transcription factor (17)
ATG4C	Important for autophagosome formation (39, 40)
ATG9A	Important for autophagosome formation (39, 40)
BECN1	Important for autophagosome formation (39, 40)
DRAM	Lysosomal protein that is a damage-regulated autophagy modulator (41)
IFNA2	Cytokine that stimulates immune cells (42)
IFNA4	Cytokine that stimulates immune cells (42)
IFNG	Cytokine that activates macrophages (38)
AMBRA1	Controls protein turnover (38); Interacts with Beclin 1-Class III PI3K complex (18)
ATG10	E2-like enzyme that is involved in the conjugation of Atg12 and Atg5 for autophagosome formation (38)
ATG16L1	Part of large protein complex necessary for autophagy (39)
ATG4A	Cysteine protease that is necessary for autophagy (38)
ATG4D	Cysteine protease that is necessary for autophagy (38)
MAP1LC3B	Subunit of the neuronal microtubule-associated MAP1A and MAP1B proteins (39)
RAB24	GTPase protein that is implicated in the regulation of intracellular protein trafficking (38)
FAM176A	Lysosome and endoplasmic reticulum-associated membrane protein (44)
ARSA	Lysosomal enzyme, arylsulfatase A, that processes sulfatides in the cell membrane (46)

(cont. on next page)

Table 11 (cont.)

EIF4G1	Involved in the initiation phase of apoptosis (38)
ESR1	Estrogen receptor important for the binding of hormones (47)
GAA	Degradation of glycogen to glucose in lysosomes (38)
PIK3C3	Important in the transport of lysosomal enzyme precursors to lysosomes (48)
PIK3R4	Involved in membrane trafficking within the endocytic pathway (38)
TMEM74	May be important for stimulating autophagy during cell stress (49)
TMEM77	Vital for p53-mediated apoptosis (50)
ULK2	Important during the activation of autophagy (45)
UVRAG	Activates Beclin 1-PI(3)KC3 complex to induce autophagy (38)
AKT1	Important for the normal functioning of the AKT-mTOR signaling pathway (51)
BAX	Binds to BCL2 to prevent BCL2's anti-apoptotic activity (38)
CDKN1B	Regulates cell cycle progression by arresting the cell cycle at G1(38)
CDKN2A	Can stop the cell cycle in the G1 and G2 phases; induces apoptosis (52)
CLN3	May be important in the proper functioning of the lysosome (46)
PTEN	Triggers cells to undergo apoptosis (46)
TGFB1	Member of the transforming growth factor family of cytokines (38)
TNF	Cytokine involved in the induction of apoptosis (53)
TP73	Part of the apoptosis response in the cell to stress (38)
BCL2	Inhibition of apoptosis autophagy pathways (18)
PRKAA1	Subunit of AMPK that can sense the cell's energy levels (38)
SQSTM1	Binds ubiquitin; regulates activation of the NFKB signaling pathway (38)
TGM2	Transglutaminase enzyme that seems to be involved in apoptosis (38)

Chapter 4: Discussion

4.1 Effects of Chlamydia pneumoniae Infection on Apoptosis and Autophagy

Previous studies have described the pathogen hypothesis of AD in which *C. pneumoniae* is implicated as a factor associated with the pathology observed in AD. In the landmark study for the pathogen hypothesis by Balin et al. 1998, *C. pneumoniae* was found to be associated with AD pathology in neuronal cells from AD brains (28). From this study, it was determined that *C. pneumoniae* DNA was more prevalent in samples of brain tissue from post-mortem AD brains than from brain tissue samples of those who did not have AD.

C. pneumoniae has been shown to manipulate its host cell's genetic machinery (28); however, presently, there are no studies that have evaluated the role that *C. pneumoniae* plays in altering the expression of genes in neuronal cells that are associated with apoptosis and autophagy. Since several studies have shown that *C. pneumoniae* inhibits apoptosis in monocytes and neuronal cells (34), we investigated *C. pneumoniae's* ability to alter the genetic regulation of apoptosis and autophagy. Apoptosis and autophagy have been recognized as key events involved in the pathology of AD (34, 21, 24). Our experimental paradigm was to determine if infection of neuronal cells by

C. pneumoniae results in changes in gene expression in the apoptotic or autophagic pathways consistent with previous work by others (21, 34) indicating that these pathways are affected in Alzheimer's disease.

This study was proposed to examine the detailed events at the level of gene expression, such as suppression of apoptosis or induction of autophagy, which may allow the neuronal cell to accumulate the cytotoxic form of β-amyloid. Since *C. pneumoniae* has been implicated as a trigger for AD and neuronal cells are thought to be a source of abnormal amyloid production, SKNMC neuronal cells were infected with *C. pneumoniae* and analyzed for changes in apoptosis and autophagy gene expression using RT^2-PCR arrays. Staurosporine, an initiator of apoptosis, was utilized to differentiate if *C. pneumoniae* is solely suppressing apoptosis gene expression or alternatively increasing autophagy gene expression.

4.2 Apoptosis Genes

The general trend observed for the key apoptosis genes was an initial down-regulation of gene expression at 24hrs followed by an increase in gene expression at 72hrs. For the genes involved in the mitochondrial pathway of apoptosis, BAK1 and BNIP3 (37), this trend indicates that apoptosis through the mitochondrial pathway may be suppressed at 24hrs and then activated by the cell at 72hrs. In contrast, the FAS gene was up-regulated at 24hrs and 72hrs. The FAS gene encodes the death receptor that interacts with proteins to initiate apoptosis (38), so the results demonstrate that the FAS gene may have been up-regulated in order to initiate the apoptosis pathway following the

C. pneumoniae infection. The CASP8 gene encodes a protein that is down-stream of the FAS receptor in the death receptor pathway of apoptosis and was down-regulated at 24hrs and 72hrs; one might speculate that the death receptor signaling pathway may not be activated at the protein level to carry out apoptosis.

The NFKB1 gene encodes a protein that is anti-apoptotic and is a nuclear transcription factor (17). In other cell lines, such as monocytes, the expression of the NFKB1 gene was increased following infection with *C. pneumoniae* (Dr. B.J. Balin and Charlie Lim MS, unpublished observations). However, in the neuronal cells, there was a decrease in expression of the NFKB1 gene at both 24hrs and 72hrs. Since the NFKB1 gene encodes a nuclear transcription factor, a decrease in NFKB1 gene expression in neuronal cells may lead to the shutdown of other genes that could assist the neuronal cell in eliminating the *C. pneumoniae* infection.

4.3 Apoptosis Genes Following Staurosporine Treatment

Following treatment of infected neuronal cells with 1μM staurosporine to induce apoptosis, the key apoptosis genes were up-regulated at 24hrs, which was followed by a decrease at 72hrs. The genes for both the death receptor and mitochondrial pathways of apoptosis were up-regulated at 24hrs, which suggests that the neuronal cells may have been stimulated to undergo apoptosis through each signaling pathway. The decrease in expression after treatment with staurosporine at 72hrs post-infection may be the result of *C. pneumoniae* suppressing apoptosis in order to maintain the infection in the host. The

NFKB1 gene encodes a transcription factor that can also be anti-apoptotic and was up-regulated at both 24hrs and 72hrs post-infection and after staurosporine treatment. These results further indicate that apoptosis may have been inhibited after 72hrs by the *C. pneumoniae* infection.

4.4 Autophagy Genes

Since the apoptosis and autophagy pathways are connected, the genes involved in autophagy were also screened following infection with *C. pneumoniae* for 24hrs and 72hrs. The general trend for the key genes implicated in autophagy was a down-regulation of gene expression at 24hrs followed by an up-regulation of gene expression at 72hrs. The ATG9A and BECN1 genes are both involved in the formation of autophagic vacuoles, and the DRAM gene is involved in the linking of the autophagosome to the lysosome (43). These results indicate that autophagic vacuole formation and linking of the autophagosome and lysosome may be suppressed at 24hrs, but are induced after 72hrs infection with *C. pneumoniae*. The ATG4C gene encodes a protein with multiple functions; specifically autophagic vacuole formation, protein targeting to the membrane/vacuole, protein transport, and protease activity. The up-regulation of the ATG4C gene may indicate that the cell is trying to induce any one of these autophagy related functions following infection with *C. pneumoniae* for 72hrs.

4.5 Genes Involved in Autophagy Induction by Intracellular Pathogens

The IFNA2, IFNA4, and IFNG genes are involved in autophagy induction when an intracellular pathogen, such as *C. pneumoniae*, has infected a cell (43). The general trend for these autophagy genes is up-regulation of gene expression at 24hrs followed by an even greater increase in gene expression at 72hrs, with the exception of the IFNA2 gene, which was down-regulated at 24hrs. This data demonstrates that at 24hrs there may be slight induction of autophagy, and at 72hrs autophagy induction may be further increased to try and eliminate the *C. pneumoniae* infection.

4.6 Autophagy Genes Following Staurosporine Treatment

Previous studies have shown that induction of apoptosis with staurosporine following infection with *C. pneumoniae* actually resulted in an inhibition of apoptosis, indicating that *C. pneumoniae* can control this process (34). Therefore, our experiments were designed to induce apoptosis with 1µM staurosporine after infection with *C. pneumoniae* to see if autophagy would be initiated instead. The general trend following staurosporine treatment for the key autophagy genes, which include ATG4C, ATG9A, BECN1, and DRAM, was an up-regulation of gene expression at 24hrs and 72hrs, with greater expression at 24hrs. The autophagic vacuole formation, linking of the autophagosome to lysosome, and protease activity functions of the key autophagy genes appeared to be induced at 24hrs and 72hrs. The addition of staurosporine to the infected neuronal cells resulted in a decrease in regulation of the key apoptosis genes and up-regulation of the key autophagy genes at 72hrs. The data from the 24hr and 72hr time

points indicates that treatment of infected neuronal cells with staurosporine may eventually suppress apoptosis, and induce autophagy instead. From these experiments, we can speculate that a *C. pneumoniae* infection may be utilizing autophagy for the survival of the cell.

4.7 Autophagy Machinery Components

There were multiple autophagy genes included in the RT^2-PCR arrays that were either associated with autophagy machinery components, which are genes implicated in the formation and clearance of autophagic vacuoles, or with genes involved involved in the regulation of autophagy. Following infection with *C. pneumoniae*, a majority of the genes that were related to the formation and clearance of autophagic vacuoles were up-regulated at 24hrs and 72hrs, with the exception of the AMBRA1 and ATG10 genes, which were down-regulated at 24hrs. The AMBRA1, ATG16L1, and MAP1LC3B genes are involved in autophagic vacuole formation. The ATG4A and ATG4D genes are associated with protein targeting to the membrane/vacuole, protease activity, and protein transport. The ATG10, ATG16L1, and RAB24 genes are also involved in protein transport. The FAM176A gene is implicated in the linking of the autophagosome with the lysosome. The general trend of up-regulation of gene expression at 24hrs and 72hrs indicates that there may have been an induction of autophagic vacuole formation, protein targeting to the membrane/vacuole, protease activity, protein transport, and linking of the autophagosome to the lysosome. However, the AMBRA1 and ATG10 genes were down-

regulated at 24hrs, which may indicate that these genes were slower to respond to the infection.

4.8 Regulation of Autophagy

The genes related to the regulation of autophagy are either involved in autophagy in response to other intracellular signals, the co-regulation of autophagy and apoptosis, or the co-regulation of autophagy and the cell cycle. There are several genes associated with autophagy in response to other intracellular signals. Following infection of the neuronal cells with *C. pneumoniae* for 24hrs, approximately half of these genes were down-regulated, while the other half of the genes in this group were up-regulated. All of these genes were up-regulated after infection for 72hrs, except for the ULK2 gene. The results at 24hrs indicate that the up- and down-regulation of these genes was offset so that autophagy may not have been induced due to interference from other intracellular signals. At 72hrs, the upward trend in gene regulation demonstrates that autophagy may have been induced in response to other intracellular signals occurring in the cell. One such intracellular signal may be low nutrient levels in the cell, such as amino acids and glucose (20).

Following infection of the neuronal cells with *C. pneumoniae*, the genes involved in the co-regulation of autophagy and the cell cycle were up-regulated at 24hrs and at 72hrs, with the exception of the CDKN1B gene and the TP73 gene at 72hrs. These results indicate that at 24hrs the infection may be influencing the cell cycle and

autophagy. Based on the data from 72hrs, there appeared to be a balance in the regulation of autophagy and the cell cycle resulting in very little change.

A majority of the co-regulators of autophagy and apoptosis were up-regulated at 24hrs and 72hrs following infection of the neuronal cells with *C. pneumoniae*, with the exception of the AKT1 gene at 24hrs and the TP73 gene at 72hrs. For most of these genes, there was greater up-regulation at 24hrs than at 72hrs. The BAX, TNF, and TP73 genes are involved in the co-regulation of apoptosis, whereas the AKT1, CLN3, PTEN, and TGFB1 genes are involved in autophagy. The data suggests that at 24hrs apoptosis may have been induced in the cell, but at 72hrs there may have been suppression of apoptosis because the TP73 gene was greatly down-regulated. In contrast, there may have been some suppression of autophagy at 24hrs since the AKT1 gene was down-regulated, although our data indicates that the autophagy genes may have been induced at 72hrs.

4.9 Co-regulation of Autophagy and Apoptosis

In the RT^2-PCR array, there were many genes involved in the co-regulation of autophagy and apoptosis; however, we found the most interesting genes in this group to be BCL2, PRKAA1, SQSTM1, and TGM2. In this instance, the co-regulation of autophagy and apoptosis refers to genes that encode proteins, which could have an effect on both the autophagy and apoptosis pathways. For instance, the protein encoded by the BCL2 gene inhibits autophagy through the inhibition of the Beclin1 protein; and inhibits apoptosis by inhibiting the BH3-only proteins, such as Bad and Bnip3 (18). Following

infection of the neuronal cells with *C. pneumoniae*, these genes were down-regulated at 24hrs and up-regulated at 72hrs. The results indicate that both autophagy and apoptosis may have been suppressed at 24hrs, especially apoptosis since the expression of the SQSTM1 gene was significantly decreased. The data shows that autophagy and apoptosis may be induced at 72hrs. However, the BCL2 gene is slightly up-regulated at 72hrs and the SQSTM1 and TGM2 genes, which encode proteins that have more of an effect on apoptosis (38), had greater up-regulation than the other genes in this group. These results may indicate that apoptosis may have been more strongly induced than autophagy at 72hrs, although our data analysis for other genes does not support this conclusion. The RT^2-PCR arrays analyze the level of gene expression, not protein expression, so protein analysis would have to be performed to determine the effects of *C. pneumoniae* on apoptosis and autophagy with regards to the co-regulatory proteins encoded by these genes.

4.10 Co-regulation of Autophagy and Apoptosis Following Staurosporine Treatment

In order to differentiate the effects of *C. pneumoniae* in neuronal cells on the key genes involved in the co-regulation of autophagy and apoptosis, the infected neuronal cells were treated with 1µM of staurosporine to induce apoptosis. At 24hrs, the BCL2 and PRKAA1 genes were up-regulated, whereas the SQSTM1 and TGM2 genes were down-regulated. A majority of these genes were up-regulated at 72hrs, except for TGM2. The up-regulation of gene expression for PRKAA1 and BCL2 suggests that following infection with *C. pneumoniae* and staurosporine treatment there may have been an

induction of autophagy at 24hrs and 72hrs with a slight decrease of induction at 72hrs. Based on the data for the SQSTM1 and TGM2 genes, apoptosis may have been suppressed at 24hrs and at 72hrs post-infection and after staurosporine treatment.

4.11 Conclusions

Autophagy and apoptosis are complex cellular processes that can enhance a cells survival or efficiently induce cell death in a controlled manner as a response to stimuli such as a bacterial infection to evade an inflammatory response. The autophagy and apoptotic processes are currently being investigated in AD as potential cellular events that may lead to neurodegeneration. In addition, there is also a complex relationship between autophagy and the endosomal/lysosomal process in Alzheimer's disease. As is demonstrated in figure 13, we speculate that following intracellular *C. pneumoniae* infection; the organism may escape autophagolysosomal and lysosomal fusion and therefore subvert degradation by lysosomal proteases creating a host friendly environment for intracellular pathogen growth within inclusions. In order for the *C. pneumoniae* to survive, the organism uses ATP produced by the mitochondria of the host cell (54), which may lead to damage of the mitochondria in the infected cell. The presence of damaged mitochondria could lead to the induction of autophagy for degradation of these damaged organelles. Our results did show an increase in the expression of genes involved in autophagy after 72hrs infection with *C. pneumoniae*, which may indicate that autophagy was induced. An increase in autophagy leads to an increase in lysosomal processing, which may lead to more endosomal-lysosomal fusion.

It has been determined that β-amyloid is produced in the endosome (12), so an increase in endosomal-lysosomal fusion may lead to increased β-amyloid production.

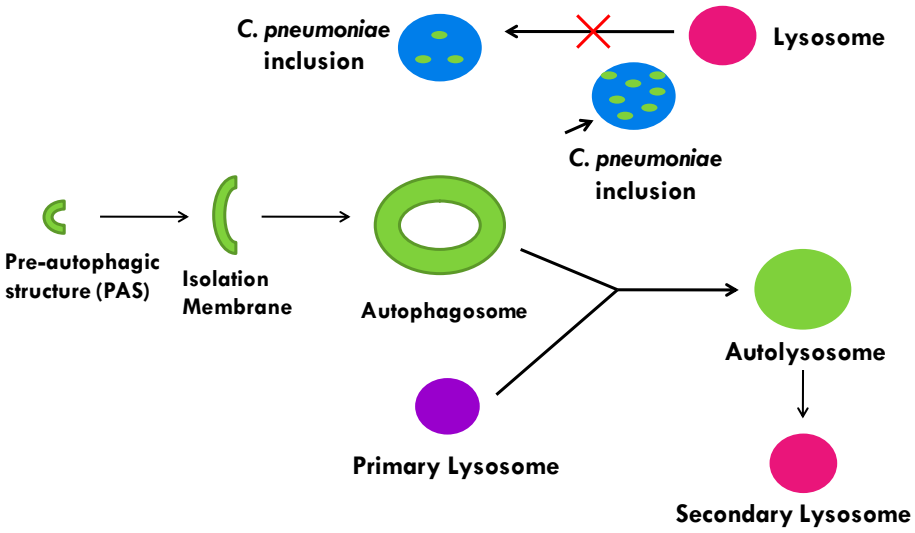

Figure 13. A model demonstrating the involvement of the autophagy pathway following C. pneumoniae infection. This model is a modification of a diagram from: Nixon, R. A. (2007). Autophagy, amyloidogenesis, and Alzheimer's disease. Journal of Cell Science, 120(23), 4081-4091. This model was proposed by Drs. Denah Appelt, Brian Balin, and Susan Hingley.

As seen in Figure 14, previous studies have shown that following infection with *C. pneumoniae* in BALB/c mice, processing of amyloid precursor protein occurred in the infected cell to produce β-amyloid (29). Infection with *C. pneumoniae* has also been shown to inhibit apoptosis (34), and the results from these experiments have demonstrated at the level of gene expression that autophagy may be inhibited initially at 24hrs post-infection, but seemed to be induced at 72hrs post-infection. The infection

with *C. pneumoniae* and possible induction of autophagy may be responsible for the production of free β-amyloid in the extracellular space between neurons. The free β-amyloid could then induce autophagy and apoptosis in neighboring cells, which may ultimately lead to neuronal cell death as seen in AD. The experiments in this study analyzed *C. pneumoniae's* effects on apoptosis and autophagy at the level of gene expression. Further investigation with experiments at the level of protein expression is in progress.

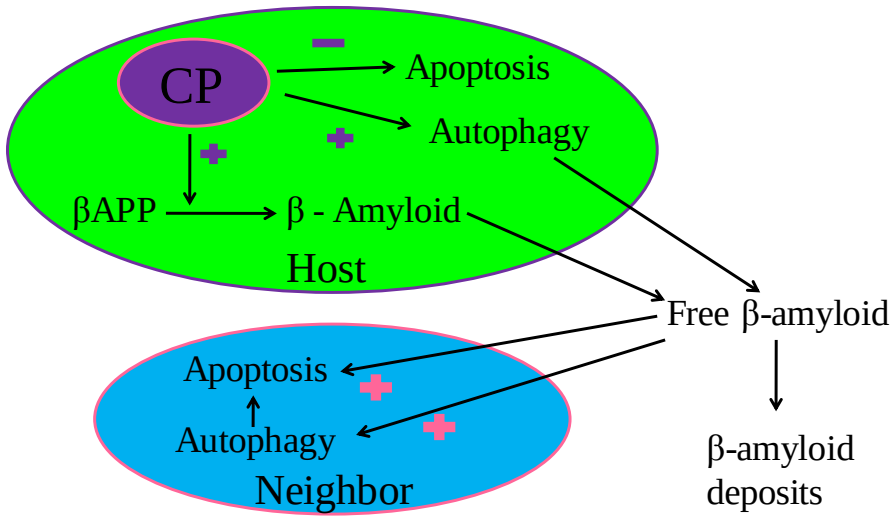

Figure 14. Proposed autophagy and apoptosis model for AD following infection with C. pneumoniae for induction of amyloid. Model proposed by Drs. Denah Appelt, Brian Balin, and Susan Hingley.

4.12 Future Directions

Future experiments need to be conducted to further prove that autophagy is induced in neuronal cells after infection with *C. pneumoniae.* It would also be interesting to observe the effects of autophagy induction and *C. pneumoniae* infection on the levels of gene expression at 48hr, 72hr, and 5 day time points to compare to the 24 and 72hr experiments profiled in this study. There are several compounds, such as rapamycin and vinblastine that induce autophagy. Specifically, treatment with vinblastine slows the fusion of autophagosomes with lysosomes, which would allow investigation of autophagy dysfunction as it applies to AD (24). Considering that the RT^2-PCR arrays only allow the analysis of gene expression levels, proteins that parallel the genes affected following infection of neuronal cells should be evaluated using immunocytochemistry and western analysis to clarify the effects on the autophagy pathway. The information gained from the proposed future experiments will enhance our knowledge of the involvement of infection in triggering the events associated with neurodegeneration seen in AD.

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