Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Philadelphia College of Osteopathic Medicine

2. **Reporting Period (start and end date of grant award period):** 1/1/2009-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Jane Z. Dumsha, PhD

4. **Grant Contact Person's Telephone Number:** 215-871-6783

5. **Grant SAP Number:** 4100047647

6. **Project Number and Title of Research Project:** 1 Dissecting the Apoptosis Pathway Affected by C. pneumoniae in Alzheimer's Disease

7. **Start and End Date of Research Project:** 1/1/2009-12/31/2012

8. **Name of Principal Investigator for the Research Project:** Denah M Appelt

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $17,098

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Appelt	Professor -PI	30% Yr 1-4
Zoga	Research Technician 1	50% Yr 1-4
Cader	Research Technician II	50% Yr 4
Reilly	Medical Student	10% Yr1
Slutter	Biomedical Masters Student	100% Yr 2
McCourt	Biomedical Masters Student	25% Yr 2
Kohler	Undergraduate: HHMI grantee	100% Yr 3-4

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes___X_____ No_____

If yes, please indicate the source and amount of other funds:
7/1/2011–6/30/2012: Internal Research Award from the Center for Chronic Disorders of Aging; $10,000

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
A new therapeutic paradigm for drug discovery	☒NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	Sept 2012	$1,229,416	$0
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify:		$	$

	_____)			

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:
These data provide preliminary results that can be used in an application for an NIH-AREA grant.

12. Future of Research Project. What are the future plans for this research project?

I plan to expand this project and study the pathways associated with Chlamydial effects on the autophagy and apoptosis pathways at the ultrastructural levels using electron microscopy and real time confocal microscopy.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1		1	
Female		2		
Unknown				
Total	**1**	**2**	**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	1	2	1	
Unknown				
Total	**1**	**2**	**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White	1	2	1	
Black				
Asian				
Other				

Unknown				
Total	**1**	**2**	**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No___X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes___X_____ No_____

If yes, please describe the collaborations:

Our development of the assays to study a pathogen's effects on cellular processes such as apoptosis and autophagy is useful in screening anti-inflammatory peptides. These novel peptides will avoid problems associated with drug induced pathogen resistance. My understanding of these cellular events is advantageous for screening the peptides and has led to my collaborative efforts with TherimuneX Pharmaceuticals, Inc.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No__X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Background

In the past few years, there has been an explosion of information and speculation as to how neurodegeneration occurs in Alzheimer's disease (AD). Alzheimer's disease is characterized by the chronic deterioration of synaptic function and a progressive loss of cortical neurons.

Cytoskeletal changes occur within cortical neurons such as the formation of paired helical filaments (PHFs) into neurofibrillary tangles (NFTs), the signature pathologies of AD. Accompanying these structures are extracellular deposits of β-amyloid, in particular the Aβ-1-42 peptide that provides a nidus for the formation of senile plaques (SPs). Prior studies have focused on the deposition of amyloid into SPs and/or the formation of PHF and NFTs; however, recently, neuroinflammation is being considered as a key factor in the pathogenesis of AD. The initiating event or stimulus for amyloid deposition, PHF/NFT formation, and neuroinflammation is still being investigated in AD.

Also being investigated as one of the primary mechanisms leading to neuronal cell death in Alzheimer's disease is apoptosis, which is a highly regulated cell death process involving activation of caspases, degradation of cytoplasmic and nuclear proteins, condensation of the nucleus, DNA fragmentation, cytoplasmic blebbing, and eventually cell death. Apoptosis is also characterized by mitochondrial dysfunction, a pathology that has been observed in AD brains. Apoptosis is not necessarily congruent with a slow progressive neurodegenerative disease, thus it is plausible that neuronal cell death may occur through several mechanisms, even though neurodegeneration itself may be initiated along with early apoptotic events. Typically, apoptosis is a common pathway by which infected cells, incapable of eliminating the infectious agent, undergo cell death; however, its completion appears to be suppressed in *Chlamydia pneumoniae* infected neuronal cells. The aims of this proposal were designed to elucidate key components of the apoptotic pathway in the neuron as they pertain to the insult of infection by *Chlamydia pneumoniae*.

The specific aims and study design of this proposal were directed by our hypothesis that a pathogen such as *Chlamydia pneumoniae* stimulates β-amyloid processing or production in neuronal cells, leading to synaptic dysfunction, neuroinflammation and apoptosis in neighboring cells, while the infected host is paradoxically protected from apoptosis.

The goal of this project is to dissect apoptotic pathways that appear to be important in both *Chlamydia pneumoniae* infection and the pathogenesis of AD and that could render the cell resistant to apoptosis.

Hypothesis: *Chlamydia pneumoniae* mediates a cellular process that blocks the mitochondrial cytochrome c release which would inhibit apoptosis and maintain an infection in the host cell.

Specific Aim 1: To assess the effects of *Chlamydia pneumoniae* infection on the mitochondrial cytochrome c pathway in neuronal cells.

Specific Aim 2: To assess the effects of *Chlamydia pneumoniae* infection on an alternate pathway that does not involve mitochondrial cytochrome c in the modulation of apoptosis. Cytochrome c release will be blocked to differentiate the pathway utilized by *Chlamydia pneumoniae.*

Results

We have previously demonstrated that *Chlamydia pneumoniae* renders the neuronal cells resistant to apoptosis; however, the exact cellular targets of *Chlamydia pneumoniae* have not

been identified in these neuronal cells. In these studies, apoptosis was experimentally induced with 1µM staurosporine for 4hrs in neuronal cells (SK-N-MC) that had been infected with *Chlamydia pneumoniae* at an MOI of 1 for 72hrs as well as in cells that had not been infected. These cells were analyzed for cytochrome c release, which is a direct consequence of mitochondrial damage by using an antibody specific to cytochrome c. The immunolabeled cells were then screened by immunofluorescent microscopy (figure 1). Cytochrome c appeared immunolocalized in a punctate pattern in uninfected neuronal cells as well as in the neuronal cells infected with *Chlamydia pneumoniae*. However, the uninfected neuronal cells that had been treated with staurosporine and were undergoing apoptosis had a more diffuse labeling pattern. These preliminary data suggest that *Chlamydia pneumoniae* may be involved in blocking cytochrome c release and/or protect the mitochondria from cellular damage.

Since immunocytochemistry is a qualitative measure of cellular events, Western analysis was utilized to determine quantitatively if caspase proteins which are activated by cytochrome c were altered in neurons infected with *Chlamydia pneumoniae* (figure 2). Neuronal cells were infected with *Chlamydia pneumoniae* for 72hrs and uninfected neuronal cells were treated with 1µM staurosporine for 4hrs and then screened by Western analysis for caspase activation using antibodies specific to caspase cleavage and activated caspase. Following incubations with staurosporine, neurons that were infected with *Chlamydia pneumoniae* exhibited a banding pattern with the caspase antibodies that was diminished from the robust banding pattern from neurons that had not been infected. As a confirmation of the *Chlamydial* infection within the neuronal cell, cell lysates were screened with an anti-*Chlamydial* antibody. Caspase activity was at basal levels in neurons that had not been exposed to staurosporine in both the infected and uninfected neurons. These data suggest that a 72-hour infection with *Chlamydia pneumoniae* in neuronal cells directly or indirectly inhibits a cascade of catalytic activation of caspases at the onset of apoptosis.

To further investigate changes in apoptosis related proteins within our experimental paradigm, studies were performed using human apoptosis antibody arrays (RayBio®; AAH-APO. These protein arrays are a nitrocellulose-based membrane system spotted with 43 antibodies that recognized apoptosis related proteins. This technology allows us to focus on those proteins that appear to be altered in *Chlamydia pneumoniae* infected neuronal cells. These studies focused on the 72hr infection with *Chlamydia pneumoniae* in neuronal cells and apoptosis was induced with 1µM staurosporine for 4hrs (figure 3). The results of this apoptosis antibody array revealed that an infection with *Chlamydia pneumoniae* in neuronal cells does lead to alterations in several pro- and anti- apoptotic proteins. Proteins that are involved either directly or indirectly with the cytochrome c pathway, such as the BCL family, caspases and cytochrome c, were decreased in CPn infected cells as compared to uninfected cells. These data confirm our hypothesis that cytochrome c pathway following a *Chlamydial* infection has a physiological relevance in the apoptotic cascade implicated in AD, as outlined in specific aim 1.

The focus of specific aim 2 was to assess the effects of *Chlamydia pneumoniae* infection on an alternate pathway that does not involve mitochondrial cytochrome c in the modulation of apoptosis. There is little doubt that neuronal cell death in Alzheimer's disease occurs through several mechanisms, and in some instances, cells may activate the autophagy pathway instead of, or before, initiating apoptosis. Intracellular constituents associated with both apoptosis and

autophagy have been identified within the Alzheimer brain. Since there are several direct correlations in the pathways between cellular mechanisms initiating both apoptosis and autophagy, we initiated RT-PCR microarray experiments evaluating genes associated with these pathways following an infection with *Chlamydia pneumoniae* for 24, 48, and 72hrs at an MOI of 1 in SKNMC neuronal cells.

To address if an infection with *Chlamydia pneumoniae* impacts the regulation of genes associated with apoptosis or autophagy, SKNMC neuronal cells (ATCC) were infected with the AR39 strain of *Chlamydia pneumoniae* at a multiplicity of infection (MOI) of 1 for 24hr, 48hr, or 72hr. Total RNA was harvested from uninfected and infected neuronal cells using an RNeasy Plus Mini Extraction Kit (Qiagen). Cell profiles were screened for gene changes related to apoptosis and autophagy using pathway-focused RT-PCR array systems (RT^2 Profiler PCR Array human Apoptosis PAHS-084Z SABiosciences). The arrays were analyzed using an Applied Biosystems 7000 Real-Time PCR machine. Comparative data analysis to determine relative expression differences between the neuronal cells infected at 24, 48 and 72hrs as compared to uninfected was performed via the $\Delta\Delta C_t$ method using the PCR Array Data analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). All assays were performed at a minimum of three runs to assure accuracy and reliability in the analysis. Relative expression levels for each gene are plotted comparing the uninfected and infected neuronal cells at each time point.

These results indicate that *Chlamydia pneumoniae* can both stimulate and inhibit gene regulation relevant to apoptosis and autophagy in an acute infection. This type of response can lead to a dysregulation of a normal cellular response to an infection such as apoptosis or autophagy. As demonstrated in the RT-PCR profiles (figure 4), many of the genes associated with apoptosis and autophagy (table 1)to remain near baselinelevels of expression at all time points. This seems to indicate that *Chlamydia* may suppress the initiation of both of these cellular events. Several gene; however, demonstrate both up- and down-regulation from 24 to 72hrs. For example, BNIP3 expression is upregulated at 48hr and is down regulated significantly at the 72hr time point. This effect may indicate that Chlamydia is altering cellular processes thereby suppressing aspects of the mitochondrial pathway. Another pair of intriguing genes is TP53 and TP73. TP53 is known to play an important in the induction of apoptosis. TP53 down-regulation starts at nearly 7-fold at 24hrs but moves closer to baseline at 48hr and 72hr where it is 2-fold down-regulated. TP73 however shows an opposite trend as it moves from 3-fold up-regulation at 24hr to 25-fold down-regulation. BCL2 , a major c- regulator of both apoptosis and autophagy remains around 2 fold up and down from 24 to 72hr, but then increases significantly at 72hrs. This gene profile may suggest that apoptosis is inhibited but autophagy is induced as is observed in neurodegenerative diseases. Similarly, the up-regulation of ATG3, ATG4C and BECN1 would suggest that autophagy may be upregulated as a result of the infection at 72hrs. Infection with *Chlamydia pneumoniae* has resulted in elevated β-amyloid peptide formation, which is a major pathology observed in AD (figure 5). Intriguingly, amyloid has been shown to activate caspases, which are also activated in apoptosis as well as in autophagy. Autophagy has been linked to Alzheimer's pathogenesis through its merger with the endosomal-lysosomal system, which also has been shown to play a role in aberrant amyloid processing. The exact role and level of autophagy regulation in the pathogenic mechanism of Alzheimer's disease has yet to be determined. Studies have suggested that aberrant autophagy induction may result in an

accumulation of autophagic vacuoles containing amyloid-β leading to the pathogenesis seen in Alzheimer's disease. Infections with *Chlamydia pneumoniae* escape autophagolysosomal fusion and therefore subvert lysosomal degradation creating a host friendly environment for the intracellular pathogen. The focus of these studies is to determine if there is a relationship interconnecting protein processing and genes regulated in autophagy following *Chlamydia pneumoniae* infection in neuronal cells.

For the infection process, neuronal cells, SKNMC (ATCC, HTB-10), were plated at $3X10^4$ to $1x10^5$ on round 18mm coverslips (Fisher, 12-545-100) for immunofluorescence or $1x10^5$ for westerns in 12 well plates (Bioexpress, T-3026-6) in total volume of 500µl growth media (ATCC, Eagle's Minimum Essential Medium 30-2003+10%FBS). The cells were infected at an MOI=1 with *Chlamydia pneumoniae* AR39 (ATCC, 53592) and incubated at 37°C in 5% CO_2 for 24-72hrs.

For immunofluorescence**, c**overslips with attached cells were rinsed with HBSS and fixed with Cytofix/Cytoperm (BD Biosciences, 554722) for 30 minutes at RT. Cells were rinsed with PBS pH 7.4 / blocked with Perm/Wash (BD Biosciences, 554723) for 30 minutes at RT and rinsed with PBS. Cells were incubated with unconjugated or conjugated antibodies for 1hr at 37°C, rinsed in PBS. The unconjugated 1° antibodies were incubated with 2° antibodies for 1hr at 37°C, rinsed in PBS. Following PBS, coverslips were rinsed in distilled water, mounted with Fluoro-Gel II Mounting Media containing DAPI (EMS,17985-50) onto slides. primary antibodies used were Lamp1(Abcam: Ab25630), Beclin-1 (Cell Signaling : 3738S), Aβ amyloid (6E10: Covance:SIG-39320) and Chlamydia pneumoniae (60-C19:Fitzgerald). Secondary antibodies used were Alexa Fluor 594 Goat Anti-mouse and rabbit Rhodamine (Invitrogen)

For Westerns analysis, cells lysates were prepared with Ripa buffer. Equal amounts of protein, as determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL), were subjected to electrophoresis through 10-20% gradient polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membranes (Invitrogen, IB3010-02). The blots were blocked with PBS/Casein (Biorad, 161-0783) for 30 minutes at RT and incubated with primary antibodies for 24hrs at 4^oC, rinsed with PBS, and blocked with PBS/Casein for 30 minutes. The blots were incubated with secondary antibodies for 2hrs RT before processing with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34080) and captured with the Kodak Imaging System. 1° antibodies used were Beclin-1 (Cell Signaling : 3738S), ATG7 (Cell Signaling: 2631), ATG12 (Cell Signaling: 2010), and *Chlamydia pneumoniae* (10C27A:Fitzgerald). 2° antibodies used were HRP Goat Anti-mouse IgG.

Cells were analyzed by immunocytochemistry and Western immunoblotting using antibodies targeting *Chlamydia pneumoniae* and autophagy proteins, such as Lamp1, Beclin-1, Atg7, and Atg12. Immunocytochemistry and Western blot analyses indicate that autophagy was induced from 24 to 72 hrs post-infection in neuronal cells. By immunocytochemistry, punctate Lamp1 labeling was localized principally in the axonal processes of uninfected cells as compared to infected cells at 24-72hrs post-infection; punctate labeling in infected cells was observed most predominantly in the cell's perikaryon at 72hrs post-infection (figure 6). *Chlamydia pneumoniae* infected neuronal cells exhibited an increased immunostaining with Beclin-1 relative to the minimal labeling observed in uninfected cells at 72hrs post-infection (figure 7). By Western blot

analysis, at 48hrs post-infection, there was an increase in immunolabeling with Beclin-1, Atg7, and Atg12 as compared to the uninfected neuronal cells (figure 8).

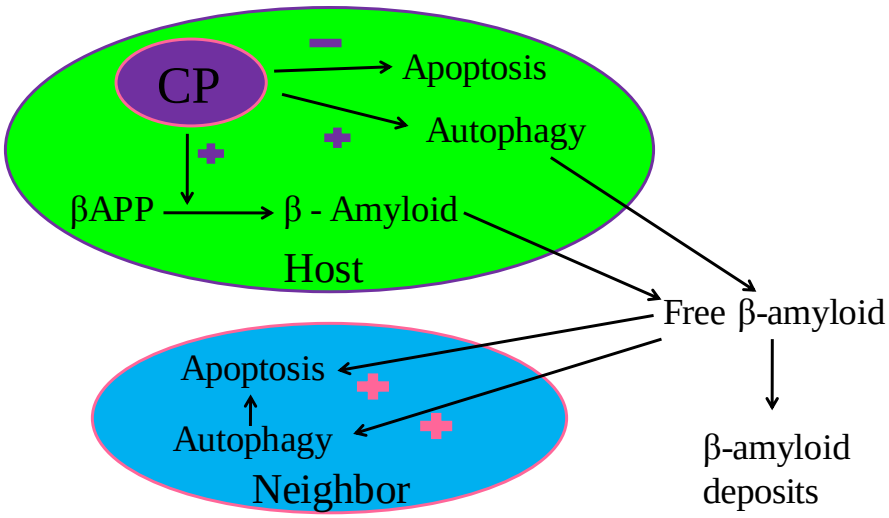
We have demonstrated that *C. pneumoniae* is capable of inhibiting apoptosis in neuronal cells, thereby prolonging the viability of the infected neuronal cell and maintaining a persistent infection. Therefore, our data give a rationale for alternative cell-death pathways such as autophagy that may be interconnected with the cytochrome c pathway associated with apoptosis. These data suggest a relationship between infection and alterations in proteins and genes common to cellular processes associated with both apoptosis and autophagy as has been observed in the pathology linked to AD.

Specific aims evaluation

The previous data generated through the support of this grant demonstrated that *Chlamydia pneumoniae* may be involved in blocking cytochrome c release and/or protect the mitochondria from cellular damage in neuronal cells. These data also indicated that an infection with *Chlamydia pneumoniae* in neuronal cells either directly or indirectly inhibits a cascade of catalytic activation of caspase at the onset of apoptosis. Therefore, these data did accomplish in year 1 of the granting period the focus of specific aim 1, which was to evaluate the effects of *Chlamydia pneumoniae* infection on the mitochondrial cytochrome c pathway in neuronal cells.

Additional experiments that were performed in Specific Aim 2 did assess the effects of *Chlamydia pneumoniae* infection on an alternate pathway that does not involve mitochondrial cytochrome c in the modulation of apoptosis. The results of these experiments redirected us into the analysis of autophagy via RT-PCR arrays that screened both apoptotic and autophagy genes. This yielded high-impact information from a set of 84 genes and will help elucidate the balance between apoptosis and the autophagic cellular process. We felt that this new direction would yield more compelling information than experiments to block release of cytochrome c release, as were originally planned for the second half of the Aim 2. In Years 2-4, the experimental focus was on analyzing the interconnection between apoptosis and autophagy and their impact on neurodegeneration observed in AD.

In summary, these studies continue to support our hypothesis that *Chlamydia pneumoniae* plays a role in "triggering" dysfunctional regulation between apoptosis and autophagy as it relates to AD pathology in neuronal cells. The chronic nature of a *Chlamydia pneumoniae* infection, with periodic reactivation of productive infection, may contribute to the gradual accumulation of pathology associated with AD. Understanding the key cellular alterations, such as the fine balance between apoptosis and autophagy and the effects that a pathogen exerts on these cellular events, is essential to the development of clinical therapeutic modalities aimed at treating or preventing neurodegenerative disease. The following diagram (model 1) is our working model integrating *Chlamydia pneumoniae* as the initiator of the alterations in cellular processes that could lead to neurodegeneration as observed in AD.



Model 1: Infection with *Chlamydia pneumoniae* alters key mechanisms in the host cell, leading to effects in neighboring cells as well as gross neuropathology.

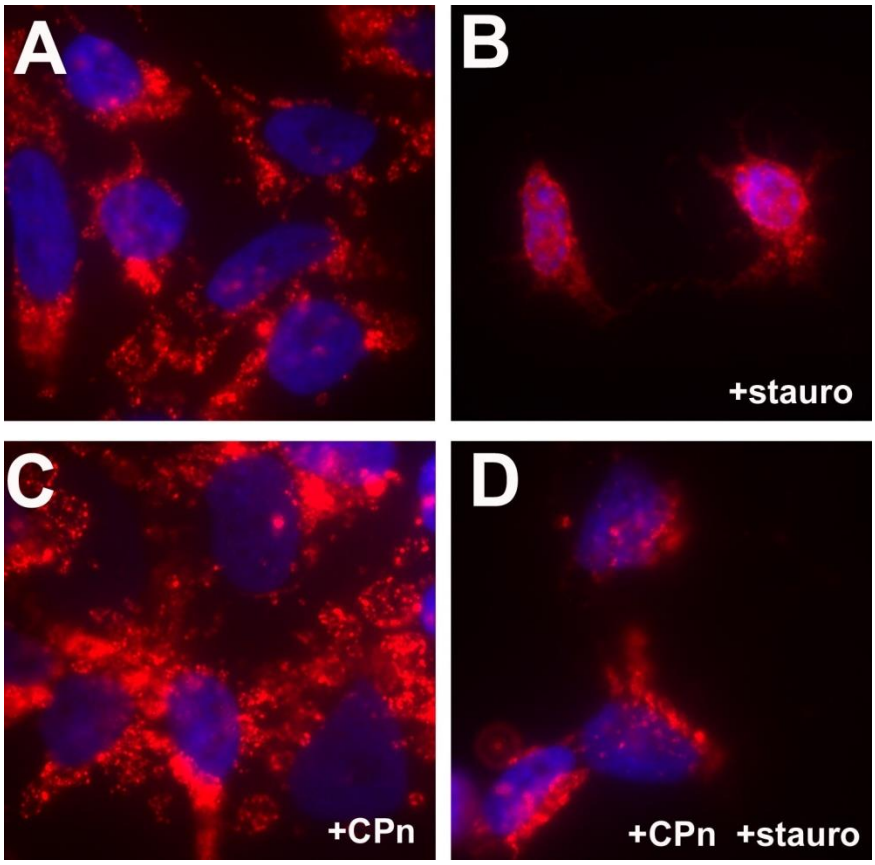


Figure 1: Human neuronal cells (SK-N-MC; HTB-10 ATCC) were infected for 72hrs with *Chlamydia pneumoniae* at an MOI of 1 followed by incubation with 1µM staurosporine for 4hrs at 37°C in 5% CO_2 and then immunoreacted for cytochrome c (red labeling; Promega #G7421). Note the punctate labeling localized to the mitochondria in neurons that were infected with *Chlamydia pneumoniae (* panel C) as compared to those neurons that were not infected (panel A). Neurons that were infected with *Chlamydia pneumoniae* and exposed to staurosporine (panel D) revealed a more punctate labeling in the mitochondria, whereas the neurons that were not infected but exposed to staurosporine (panel B), displayed a more diffuse cytochrome c immunoreactivity. The nucleus (blue) of neurons infected with *Chlamydia pneumoniae (panel C)* and treated with staurosporine (panel D) maintained a normal nuclear profile as compared to the uninfected neurons incubated with staurosporine (panel B). The nuclear fragmentation demonstrates the classic morphology associated with a cell undergoing apoptosis.

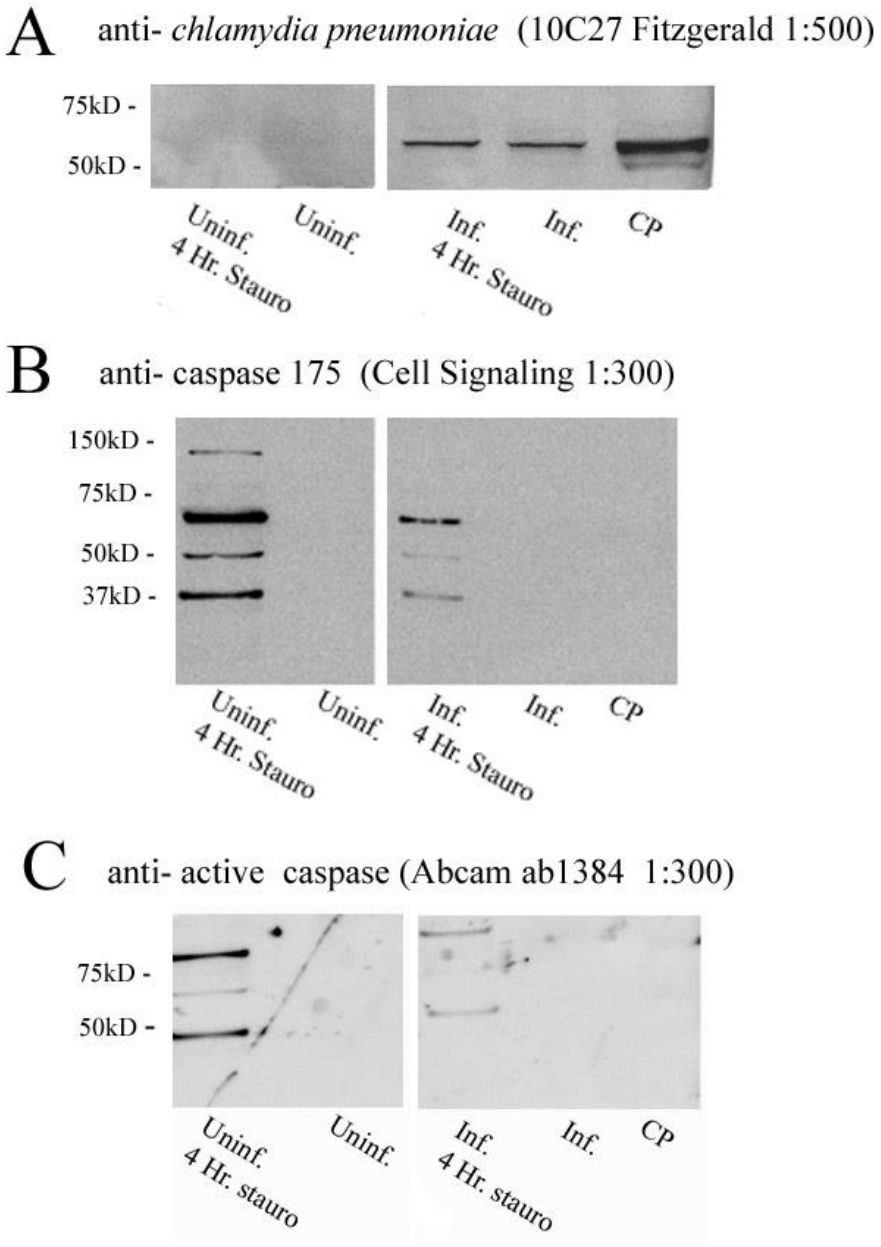


Figure 2: Representative Westerns showing caspase activation in human neuronal cells (SK-N-MC;HTB-10 ATCC) that were infected for 72hrs with *Chlamydia pneumoniae* at an MOI of 1 followed by incubation with 1µM staurosporine to induce apoptosis for 4 hrs and 6hrs. A monoclonal antibody specific for *Chlamydia pneumoniae* was used to verify the chlamydial infection from the cellular lysates of the neuronal cells (panel A). Complexes of caspase are evident in uninfected cells induced with staurosporine into apoptosis at 4 hrs (panels B and C). Cells that have been infected and treated with staurosporine appear to inhibit caspase processing as compared to the uninfected cells treated with staurosporine (panels B and C). Cells were processed in Ripa lysis buffer and 5µg to 10 µg of total protein was prepared for electrophoresis on a 10-20% Tris Glycine gel from Biorad, then transferred onto nitrocellulose membranes.

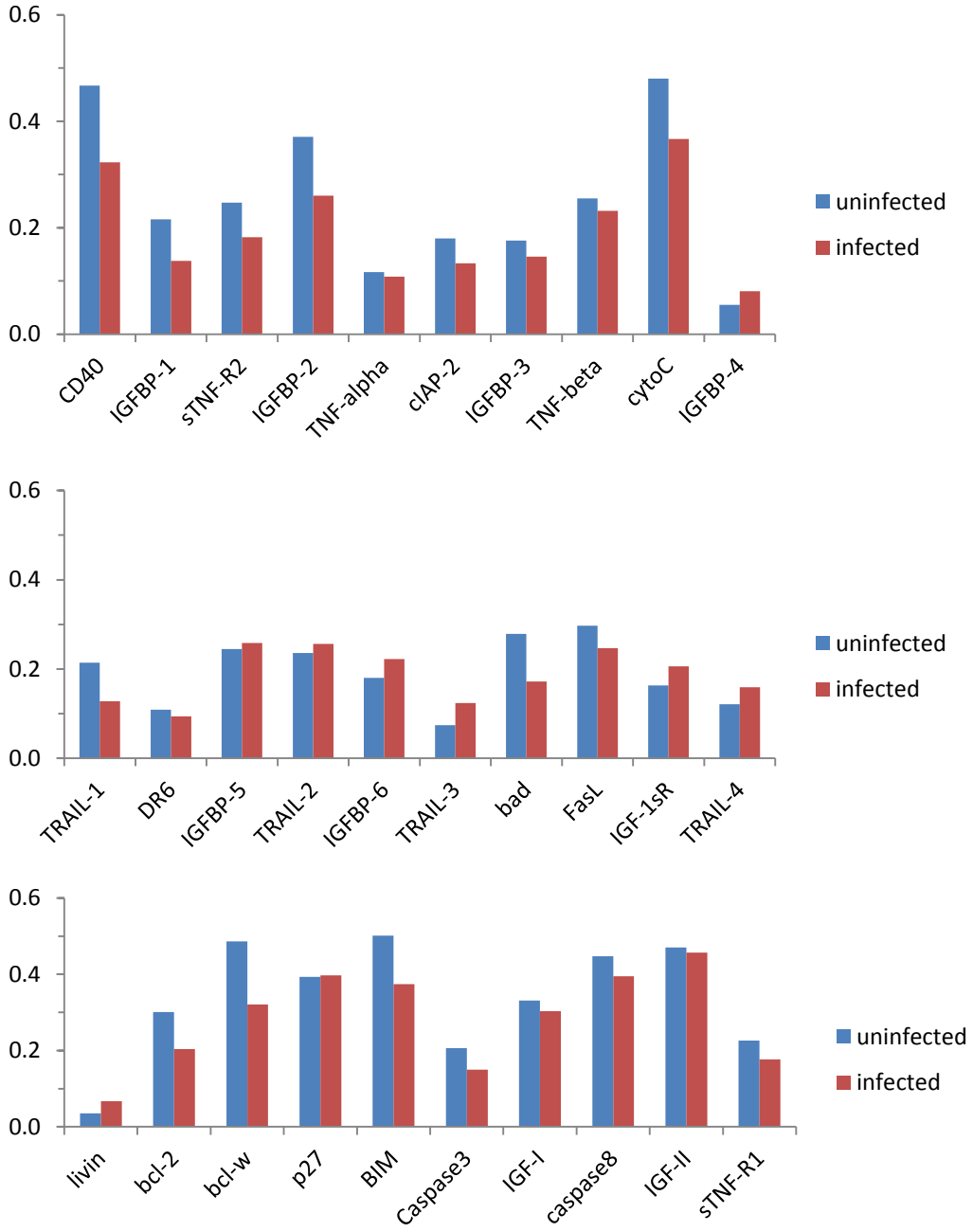


Figure 3: RayBio® human apoptosis antibody array (AAH-APO) was used to screen for apoptosis related proteins. Neuronal cells (SKNMC) were infected with *Chlamydia pneumonia*e at an MOI of 1 for 72hrs and both uninfected and infected cells were incubated with 1µM staurosporine for 4 hrs. The chlamydial infection appeared to alter several apoptotic related proteins that are both pro and anti-apoptotic. A total of 400ug of cell lysate was used for this assay.

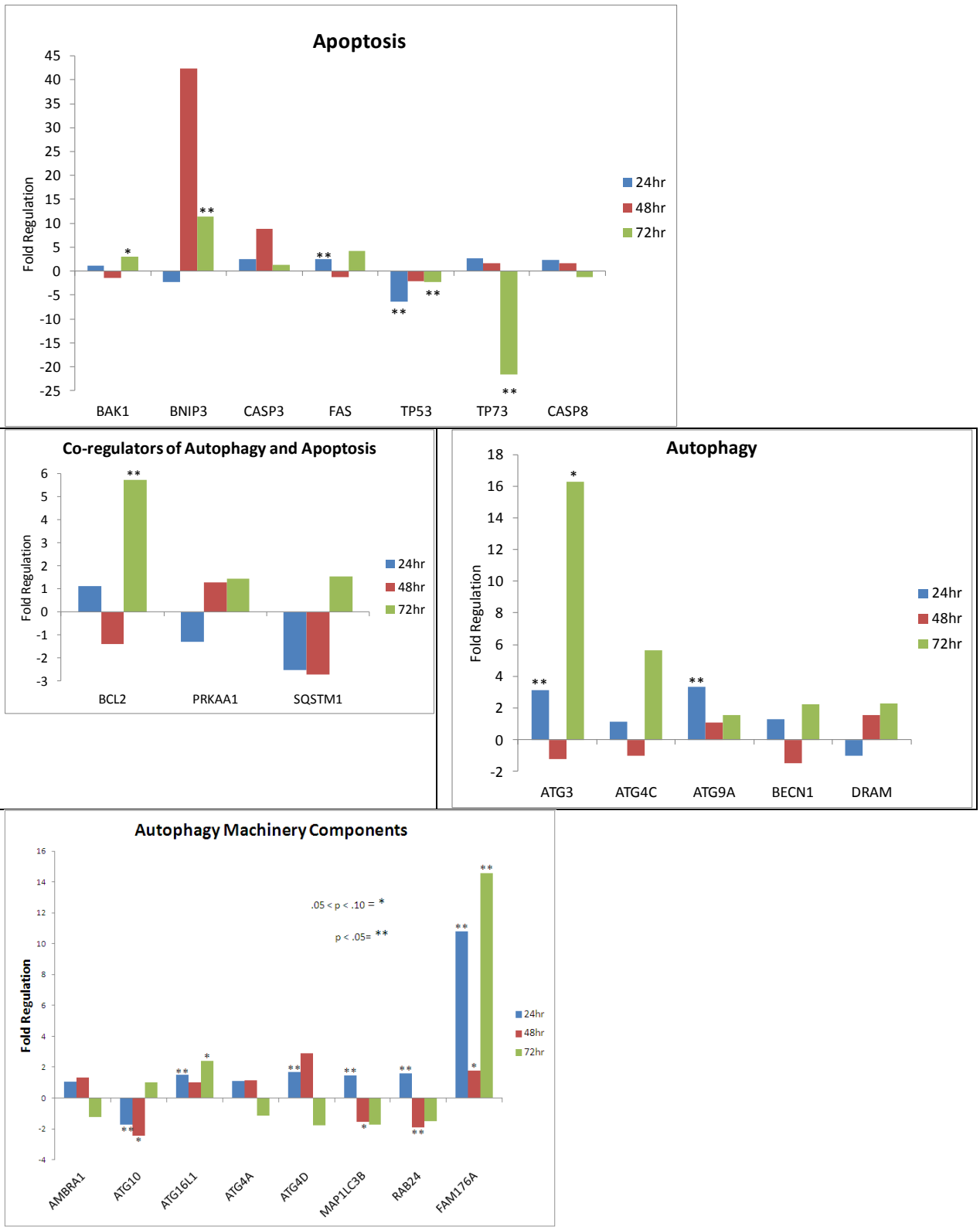


Figure4: Apoptosis and Autophagy RTPCR profile from neuronal cell infected with *Chlamydia pneumoniae* at an MOI of 1 compared to uninfected neuronal cell at 24, 48 and 72hrs. For all profiles : *p-value between 0.05 and 0.10 / **p-value less than 0.05

Table 1. Apoptosis and Autophagy genes from RTPCR profile

Gene	Functions of Gene Products
BAK1	Contributes to the mitochondrial pathway of apoptosis
BNIP3	Contributes to the mitochondrial pathway of apoptosis
CASP3	Execution phase of apoptosis
FAS	Death receptor that induces apoptosis via the death receptor pathway
TP53	Inducing cell cycle arrest and apoptosis in response to cellular stresses
TP73	homolog of p53 and may promote p53 independent apoptosis pathway
CASP8	Initiator caspase that induces apoptosis via the death receptor pathway
ATG3	Essential for autophagocytosis
ATG4C	Important for autophagosome formation
ATG9A	Important for autophagosome formation
BECN1	Important for autophagosome formation
DRAM	Lysosomal protein that is a damage-regulated autophagy modulator
AMBRA1	Controls protein turnover (38); Interacts with Beclin 1-Class III PI3K complex
ATG10	E2-like enzyme that is involved in the conjugation of Atg12 and Atg5 for autophagosome formation
ATG16L1	Part of large protein complex necessary for autophagy
ATG4A	Cysteine protease that is necessary for autophagy
ATG4D	Cysteine protease that is necessary for autophagy
MAP1LC3B	Subunit of the neuronal microtubule-associated MAP1A and MAP1B proteins
RAB24	GTPase protein that is implicated in the regulation of intracellular protein trafficking
FAM176A	Lysosome and endoplasmic reticulum-associated membrane protein
BCL2	Inhibition of apoptosis autophagy pathways
PRKAA1	Subunit of AMPK that can sense the cell's energy levels
SQSTM1	Binds ubiquitin; regulates activation of the NFKB signaling pathway

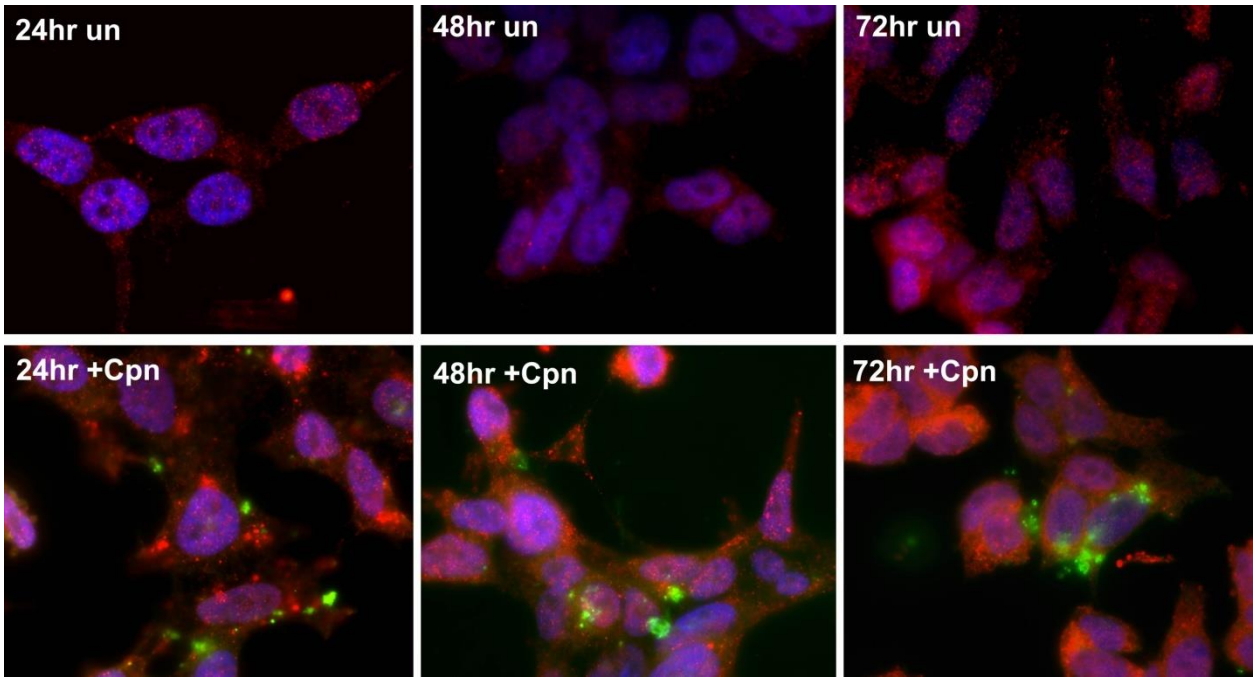


Figure 5: An increase in intracellular Aβ is observed in neuronal cells that have been infected with *Chlamydia pneumoniae* from 24 to 72hrs as compared to the uninfected neuronal cells. Aβ amyloid antibody (red), chlamydial antibody (green) and nuclei (blue).

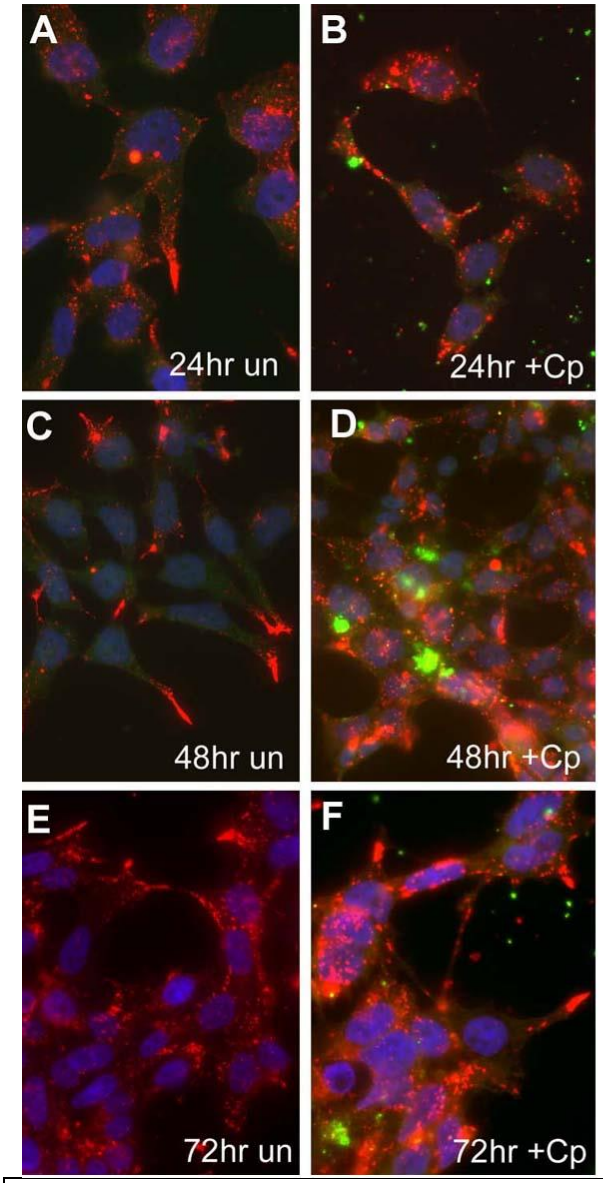


Figure 6: Neuronal cells were screened for the presence of autophagosomes following an infection with *Chlamydia pneumoniae* for 24 to 72hrs. An increase in autophagic vacuoles is observed within the cytoplasm of the infected neuronal cells that had been infected with *Chlamydia pneumoniae* (green) for 24 to 72hrs as compared to uninfected cells. Immunofluorescent labeling with Lamp1 antibody (red) was used to assess autophagic vacuoles.

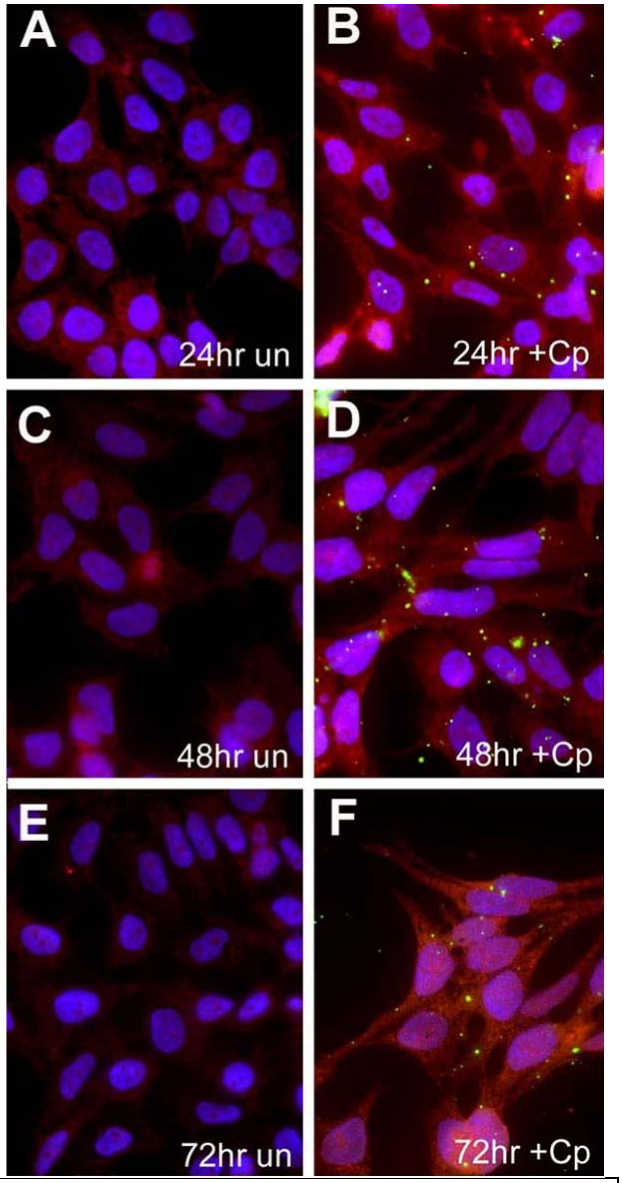


Figure 7: *Chlamydia pneumoniae* (green) appeared to induce autophagy in neuronal cells that had been infected for 24 to 72hrs as revealed by the Beclin 1(red) immunofluorescence labeling.

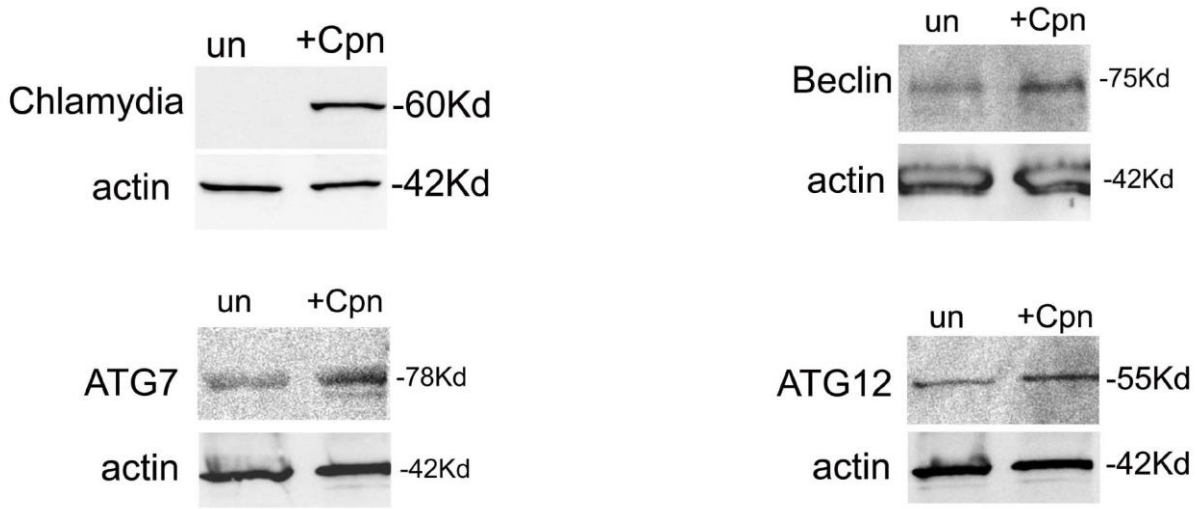


Figure 8: Western analysis of neuronal cells infected with *Chlamydia pneumoniae* for 48hrs at an MOI of 1. Infected cells demonstrated an increase in immunostaining for autophagy proteins ATG7, Beclin, and ATG12. The cells lysates were screened for the presence of a *Chlamydial* infection as observed by the 60Kd band.

<u>Abstracts presented at scientific meetings:</u>

Slutter, A., Zoga, J.D., Hingley S.T., Balin, B., Appelt, D.: Analysis by Real-Time PCR of Autophagy and Apoptosis in Neuronal Cells Infected with *Chlamydia Pneumoniae.*
*Presented at the Chlamydia Basic Research Society:*2011

Appelt, D., Slutter, A., Zoga, J.D., Hingley K., Reilly, M., Hingley, S.T., Balin, B.: Analysis of Autophagy and Apoptosis Gene Regulation in Neuronal Cells Infected with *Chlamydia Pneumoniae:*
Presented at the International Conference on Alzheimer's Disease:2011

Zoga, J.D., Slutter, A., Balin, B. Appelt, D.: Chlamydia pneumoniae induces the host autophagic pathway in neuronal cells establishing an environment for β1-42 processing.
*Presented at the Society for Neurosciences:*2011

Appelt, D., Kohler, I., Slutter, A., Zoga, J.D., Hingley, S.T., Balin, B: Autophagy and apoptotic genes implicated in Alzheimer's disease are modulated following infection of neuronal cells with *Chlamydia pneumoniae*
Presented at the International Conference on Alzheimer's Disease:2012

Kohler, I., Slutter, A., Zoga, J.D. Hingley, S.T., Balin, B., Appelt, D.: Genetic dysfunction related to Alzheimer's disease is found in neuronal cells after short-term and long-term *Chlamydia pneumoniae* infection.
Presented at the Society for Neurosciences:2012

Balin, B., Hammond, C., Zoga, J., Cader, A., Slutter, A., Anzmann, J., Kohler, I., Hingley, S., Appelt, D.: Analysis of autophagy and inflammasome regulation in neuronal cells and monocytes infected with *Chlamydia pneumoniae*: Implications for Alzheimer's disease.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X____ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
___X___ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed

publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.				☐Submitted ☐Accepted ☐Published
2.				☐Submitted ☐Accepted ☐Published
3.				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:
Within the year 2013, I plan to submit manuscripts for publication that will focus on genes associated with apoptosis and autophagy in neuronal cells followed by an infection with Chlamydia. Additionally, the manuscript will contain the data revealing the protein

dysregulation with autophagy and apoptosis that will ultimately demonstrates a pathogens involvement in neurodegenerative diseases such as Alzheimer's' disease.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
   Yes_____ No____
   If yes, indicate number of patent, title and date issued:
   Patent number:
   Title of patent:
   Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

   If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

   If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on for each person. (See attached sample). ***DO NOT EXCEED FOUR PAGES.***

NAME	POSITION TITLE
Denah Marie Appelt	Professor

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Juniata College, Huntingdon, PA.	*B.S.*	*1979*	*Biology*
University of Pennsylvania, Phila. PA.	*Post-bac*	*1987-90*	*Cell Biology*
Medical College of Pennsylvania, Phila. PA.	*Ph.D.*	*1996*	*Neuroanatomy*
Hahnemann University, Phila. PA	*Post-doc*	*1996-1997*	*Pathology & Lab Med.*

Positions and Employment

1995 Instructor: Gross Anatomy & Histology: Medical College of Pennsylvania & Hahnemann Univ., Philadelphia., PA.
1996 Instructor: Gross Anatomy; Univ. of Pennsylvania School of Medicine, Phila., PA.
1996-1997 Research Associate: Department of Path & Lab Med., Hahnemann Univ., Phila., PA.
1997-1998 Research Assistant Professor: Dept. of Path & Lab Med., Hahnemann Univ., Phila., PA.
1998-2002 Assistant Professor: Dept. of Biosciences Philadelphia College of Osteopathic Medicine, Phila., PA.
2002-2006 Associate Professor: Dept. Neuroscience, Physiology and Pharmacology, Philadelphia College of Osteopathic Medicine, Phila., PA.
2006-present Professor: Dept. of Neuroscience, Physiology and Pharmacology Philadelphia College of Osteopathic Medicine, Phila., PA.

PCOM commitments and professional development

1998-present Director; Biological Imaging : Philadelphia College of Osteopathic Medicine
1999-present DO Admissions committee
1998 – 1999 Executive Faculty at large:
2003-present Appointment, Promotion and Tenure:
2003-2005 Committee on Committees
2004 Organizing committee for Challenging Views of Alzheimer's Disease
2007-present (Co-Director)DO/PhD program and development committee
2007-present Faculty Senate -Secretary
2009-present Vice-Chair Environmental Safety Committee
2011-2012 AOA COCA committee on preclinical
2012-present Director of Clinical and Basic Neuroscience

C. Selected Peer-Reviewed Publications:

1. Balin, BJ, Gerard, HC, Arking, EJ**,** Appelt, DM, Branigan, PJ, Abrams, JT, Whittum-Hudson, JA, and Hudson, AP: Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med Microbiol Immunol*. 1998; 187(1):23-42. PMID: 9749980
2. Balin BJ, Appelt DM. Transglutaminase-catalyzed formation of alzheimer-like insoluble complexes from recombinant tau. In: Hooper NM, ed. *Methods in Molecular Medicine. Alzheimer's Disease Methods and Protocols.* Totowa, NJ: Humana Press Inc; 2000:395-403.
3. Balin BJ, Appelt DM, Whittum-Hudson JA, Hudson AP. Infectious agents and Alzheimer's disease. In: *Recent Research Developments in Molecular and Cellular Biochemistry.* Vol 1. ; 2003:187-202.
4. Balin BJ, Hammond CJ, Little CS, MacIntyre A, Appelt DM. *Chlamydia pneumoniae* in the pathogenesis of Alzheimer's disease. In: Bendinelli M, Yamamoto Y, Friedman H, eds. *Chlamydia Pneumoniae Infection and Diseases* New York, NY: Kluwer Academic/Plenum; 2004:211-226.
5. Little CS, Hammond CJ, MacIntyre A, Balin BJ, Appelt DM. Chlamydia pneumoniae induces alzheimer-like amyloid plaques in brains of BALB/c mice. *Neurobiol Aging*. 2004; 25(4):419-429. PMID: 15013562
6. Itzhaki RF, Wozniak MA, Appelt DM, Balin BJ. Infiltration of the brain by pathogens causes Alzheimer's disease. *Neurobiol Aging*. 2004; 25(5):619-627. PMID: 15172740
7. Way DS, Albert EV, Hammond CJ, Bell MG, Balin BJ, Appelt DM. Caspase activity is inhibited in neuronal cells infected with chlamydia pneumoniae: Implications for apoptosis in Alzheimer's disease. 10th ICAD & Related Disorders. *Alzheimer's Disease: New Advances*. 2006:295-299.
8. Balin BJ, Appelt DM, Little CS, Hammond CJ, Whittum-Hudson JA, Hudson AP. Chlamydophila (Chlamydia) pneumoniae in Alzheimer's disease. In: Seyfang A, Friedman H, Bendinelli M, eds. *Microbial Infections of the Neurologic System.* Springer; 2007.
9. Brian J. Balin, C. Scott Little, Christine J. Hammond, Denah M. Appelt, Judith A. Whittum-Hudson, Hervé C. Gérard, and Alan P. Hudson,. Chlamydophila pneumoniae and the etiology of late-onset alzheimer's disease. *J Alzheimers Dis*. 2008; 13(4):371-380. PMID: 18487846
10. Appelt,D, Roupas,M, Way,D, Bell,MG, Albert,E Christine J. Hammond, and Brian J. Balin:. Inhibition of apoptosis in neuronal cells infected with *chlamydophila (chlamydia) pneumoniae*. *BMC Neurosci*. 2008; 9:13. 10.1186/1471-2202-9-13. PMID: 18218130
11. Galluzzi KE, Appelt DM, Balin BJ. Modern care for patients with Alzheimer disease: rationale for early intervention. *J Am Osteopath Assoc*. 2010 Sep;110(9 Suppl 8):S37-42.PMID: 20926742
12. Hammond CJ, Hallock LR, Howanski RJ, Appelt DM, Little CS, Balin BJ. Immunohistological detection of Chlamydia pneumoniae in the Alzheimer's disease brain. *BMC Neurosci*. 2010 Sep 23;11:121.PMID: 20863379
13. Thacker, JD, Balin, BJ, Appelt, DM, Sassi-Gaha, S, Purohit, M, Rest, RF, Artlett, CM: Host-response to injury and infection and development of a first-in-class, broad-spectrum, anti-infective drug. Antimicrobial Agents and Chemo., epub Jan. 30, 2012.