

# Duquesne University

## Annual Progress Report: 2012 Formula Grant

### Reporting Period

July 1, 2013 – June 30, 2014

### Formula Grant Overview

Duquesne University received \$100,224 in formula funds for the grant award period January 1, 2013 through December 31, 2015. Accomplishments for the reporting period are described below.

### **Research Project 1: Project Title and Purpose**

*Regulators of Biofilm Formation in the Pathogenic Fungus, Candida. albicans* – The fungal pathogen, *Candida albicans*, is one of the most common hospital acquired infections worldwide. Critically ill patients, including those with various forms of cancer, are particularly susceptible to this infection. The pathogenicity of *C. albicans* is enhanced by its ability to grow on the surface of medical devices in the form of biofilms. Biofilms are complex networks of ovoid and filamentous cells that communicate with one another through secreted molecules. The purpose is to identify extracellular signaling molecules produced by *C. albicans* that regulate biofilm formation and thereby contribute to the pathogenicity of the organism.

### Anticipated Duration of Project

1/1/2013 – 12/31/2015

### Project Overview

Biofilms are complex networks of ovoid and filamentous cells that communicate with one another through secreted molecules, also known as quorum sensing molecules, to relay information about cell density and environmental conditions. To date, only a handful of those molecules have been identified, but it is widely believed that many others exist. Notably, this area of research is understudied, and no published examination of the extracellular metabolome of *C. albicans* biofilms has been reported. Our central hypothesis is that *C. albicans* produces as yet unidentified extracellular signaling molecules that regulate biofilm formation and thereby contribute to the pathogenicity of the organism.

We propose the following two specific aims to investigate our central hypothesis:

AIM 1 - Analyze the extracellular metabolites produced by *C. albicans* biofilms to identify potential regulatory molecules. We will employ a combination of mass spectrometry approaches to first profile the extracellular metabolites and then to absolutely identify molecules of interest.

We will compare the extracellular metabolites produced by planktonic cultures to those produced by biofilm cultures.

AIM 2 - Test candidate regulatory molecules for their role in biofilm formation. Metabolites identified under Specific Aim 1 will be tested for their ability to stimulate or inhibit biofilm formation using various methods, including confocal and scanning electron microscopy. In addition, reporter strains that allow for the determination of the relative amount of yeast versus hyphal cells will be employed as a way of identifying subtle changes in biofilm morphology.

### **Principal Investigator**

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### **Other Participating Researchers**

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### **Expected Research Outcomes and Benefits**

The proposed project is a collaborative effort that utilizes the expertise of two investigators at Duquesne University to attack an important health-related problem. *C. albicans* biofilms are a major source of hospital acquired infection in the US, and patients undergoing cancer chemotherapy, as well as other immunosuppressive treatments, are especially at risk. We expect to identify and test an initial set of biofilm and/or planktonic culture specific metabolites for their role in inhibiting or promoting biofilm formation in the pathogenic fungus, *C. albicans*. By identifying the metabolites that are critical in the biofilm formation/ inhibition process we can then begin to explore the uses of the molecules from a biomedical perspective to stop biofilm formation on catheters, where infections caused by *C. albicans* are critical and potentially deadly for immunosuppressed patients. For example, inhibitory molecules identified here may be infused into new polymer blends used for catheters, thereby reducing infections rates.

### **Summary of Research Completed**

The long-term goal of this project is to modulate biofilm formation by *Candida albicans* using naturally produced molecules. The hypothesis is that extracellular metabolites found in biofilm cultures are produced to either inhibit or promote cellular growth. In this segment of the project the objective is to determine the metabolites that are present in planktonic and biofilm cultures. Utilizing this information, the molecules that vary in culture, either in type or amount, will be used to modulate biofilm formation.

*Aim 1 - Analyze the extracellular metabolites produced by C. albicans biofilms to identify potential regulatory molecules. We will employ a combination of mass spectrometry approaches to first profile the extracellular metabolites and then to absolutely identify molecules of interest. We will compare the extracellular metabolites produced by planktonic cultures to those produced by biofilm cultures.*

No work was performed on this aim in this reporting period due to instrumentations difficulties.

*Aim 2 - Test candidate regulatory molecules for their role in biofilm formation. Metabolites identified under Specific Aim 1 will be tested for their ability to stimulate or inhibit biofilm formation using various methods, including confocal and scanning electron microscopy.*

In this reporting period, we took two approaches to determining the extracellular metabolites in both planktonic and biofilm cultures. This was done, in part, out of need. The initial approach was to identify extracellular metabolites by analyzing the media of planktonic and biofilm cultures of various times points via the Q-ToF mass spectrometer (Agilent 6530). However, this instrument was out of service frequently during this reporting period due to various mechanical issues. Additionally, it was determined that the instrument needed an upgrade to improve quantitation. The reference mass system was missing from the instrument and it required that we acquire and install it as well as a source of clean, compressed air. This system is still only partially installed. It should be fully installed by the end of July. The quantitation upgrade is necessary for this project because in order to understand the regulation of biofilms the quantity, not just the identity, of key metabolites in planktonic and biofilm cultures must be determined. Due to these delays, the mass spectrometric analysis of the project proceeded intermittently as described below.

Previously the media from biofilm and planktonic cultures (*WT-DAY185 C. albicans*) were analyzed by the Q-ToF mass spectrometer without separation by liquid chromatography. Significant differences were seen in the mass spectra of spent media from the formed biofilms and planktonic cultures and as compared to fresh growth media (The growth media consisted of 2% glucose, 0.5% ammonium sulfate, 75 $\mu$ M inositol, 80  $\mu$ g/mL uridine, yeast nitrogen base mix, trace salt mixture, vitamin mixture and an amino acid mixture.). The individual components of the growth media were also analyzed to determine if the changes were due to alterations of common media components such as amino acids and to definitively identify the media component peaks.

In order to improve this process, in this reporting period, an ultra high capacity small molecule chip with a 500nL enrichment column and a 150 mm x 75  $\mu$ m separation column was purchased for the 1260 Infinity HPLC-Chip/MS system for liquid chromatography purposes. The stationary phase is non-polar (Zorbax 80SB-C18). With the chip cube attached, mobile phase and instrument parameters were established using the growth media. The capillary voltage was set to 2000V while the nano pump flow rate was 0.4  $\mu$ L/minute and the capillary pump flow rate was 4  $\mu$ L/minute. The mass range was 20 to 800 m/z with a scan rate of 5 spectra/ second. The N<sub>2</sub> gas temperature was set to 300°C at 5 L/min. The mobile phase used a gradient consisting of two components mixtures A (98% H<sub>2</sub>O, 2% Acetonitrile, 0.1% Formic Acid) and B (80% acetonitrile, 20% H<sub>2</sub>O, 0.1% Formic Acid) which were then combined using a gradient (Table 1).

The MS ToF Fragmentor was set to 150V while the skimmer was set to 65V. These instrument parameters were combined with a fractionation protocol to separate the hydrophobic and hydrophilic portions of the media. Both fractions were evaluated by MS. In the hydrophobic layer, a majority of the components were eluting from the column in a 2 minute window. Optimal separation was not achieved (Figure 1) but qualitatively the data was being evaluated and new LC parameters were being developed when the instrument broke down. It has been down since February. Once the instrument is repaired experiments will continue in this vein including optimization of the separation of media components from the biofilm and planktonic cultures and the QToF settings. Once optimal separation is achieved the most recent Agilent operating software has recently been installed and will be used to help identify the varying extracellular components.

In a second approach aimed at finding bioactive molecules, we are examining the ability of extracellular secretions from biofilm and planktonic cultures to act as quorum sensing molecules for *C. albicans* prior to identifying candidate compounds through MS. Instead, we plan to fractionate the media through more traditional techniques, isolate bioactive fractions, and then positively identify the bioactive compound through MS at a later time. In concert with this approach, we may also study the effect of these fractions on macrophage activation. Importantly, these sets of experiments will allow us to make progress on identifying bioactive compounds prior to having a fully functional MS.

In order to establish appropriate experimental benchmarks, *C. albicans* response to a known quorum sensing molecule, farnesol, was tested. As a control for biofilm formation, cells were incubated overnight in growth media. Bovine serum albumin (BSA) was added to each well of a six well plate (except the control well) and was allowed to sit on the bench top for 24 hours for protein adherence. Cells were diluted to obtain an OD<sub>600</sub> of 0.5. The culture was added directly to the well plate and placed on the incubator at 35 rpm and 37°C for 24 hours. Adherent biofilms grew in the wells coated with BSA. Cells were mainly in the hyphae and pseudohyphae form (Figure 2).

To test the effect of farnesol, the well plates and culture were grown as before. Cells were diluted to obtain an OD<sub>600</sub> of 0.5 and to this culture the appropriate amount of farnesol was added to obtain a 300 µM concentration (original stock solution of farnesol was 30 mM in methanol). The culture was added directly to the well plate and placed on the incubator at 35 rpm and 37°C for 24 hours. After the 24 hour time point, the cells were observed by optical microscopy (Figure 3). The farnesol inhibited the germination of adherent cells with only yeast cells in culture. No pseudohyphae or hyphae cells were present. This result is consistent with the literature, which show that farnesol is a known biofilm inhibitor. In a separate experiment, a culture was diluted to obtain a 3µM of farnesol. The remainder of the experiment was identical to those above. At this low concentration, farnesol did not inhibit biofilm formation, which is also consistent with the literature.

Now that the biofilm growth conditions for this type of evaluation have been set and literature precedence has been repeated, the ability of the contents of the media surrounding biofilms (biofilm media) and the media from planktonic cultures (planktonic media) to effect growth behavior will be evaluated. In preliminary experiments, the cells were incubated overnight in

growth media. BSA was added to each well (except the control well) and was allowed to sit on the bench top for 24 hours for protein adherence. Cells were diluted with biofilm media to obtain an OD<sub>600</sub> of 0.5. Biofilm media is obtained by centrifuging the media and well plate contents in which the biofilms grew. The supernatant is then diluted 1:1 with fresh growth media and stored at 4°C until use. This media, therefore, should contain any extracellular metabolites from cells in biofilms. Cells were then grown in the biofilm media in a six well plate and placed on the incubator for 24 hours at 35 rpm and 37°C. Biofilm formation was reduced but results were mixed, therefore this work must be repeated.

Similarly, cells were grown in planktonic media. Planktonic media is obtained by centrifuging the media from the planktonic growth. In this way, the media would contain extracellular metabolites from motile cells. The supernatant is then diluted 1:1 with fresh growth media and stored at 4°C until use. Cells were then grown in the planktonic media in a six well plate and placed on the incubator for 24 hours at 35 rpm and 37°C. The preliminary results were mixed and therefore will be repeated.

If an effect is seen from either the media extracted from biofilm or planktonic media, then the media will be further fractionated appropriately. The fractions will be analyzed by mass spectrometry to identify the components and utilized in the biofilm forming assays above to narrow the fraction, which contains the effective extracellular metabolites.

Table 1. LC Gradient

<b>Time</b>	<b>%B</b>
0	2
2	5
4	10
7	30
10	60
13	80
16	90
19	100
24	2
26	2

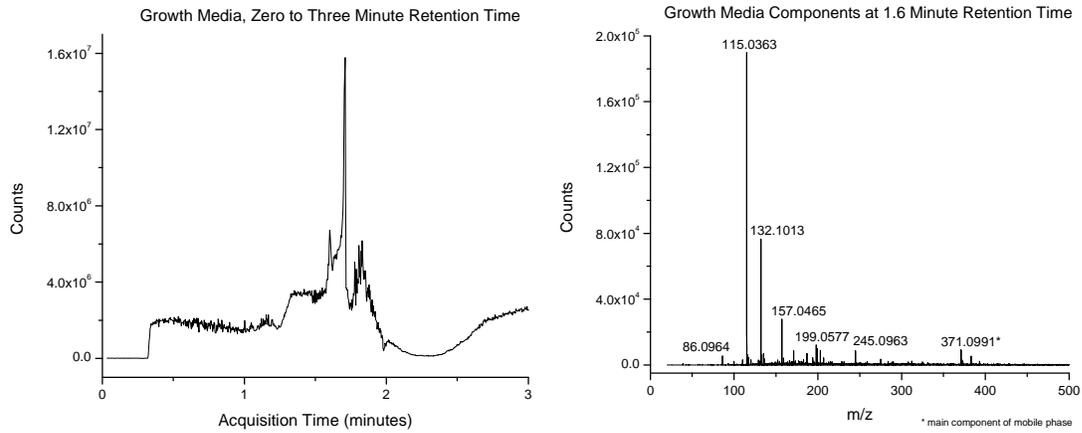


Figure 1.

Figure 1. Sample MS from growth media using the LC/MS settings described above. One of the peaks at 1.6 minutes was determined to be leucine and confirmed by the METLIN Database. Leucine = 132.1013 m/z [M + H]<sup>+</sup> (Actual mass = 131.17 g/mol)



Figure 2. *C. albicans* cells in hyphae form in biofilm after 24 hours of growth on BSA.

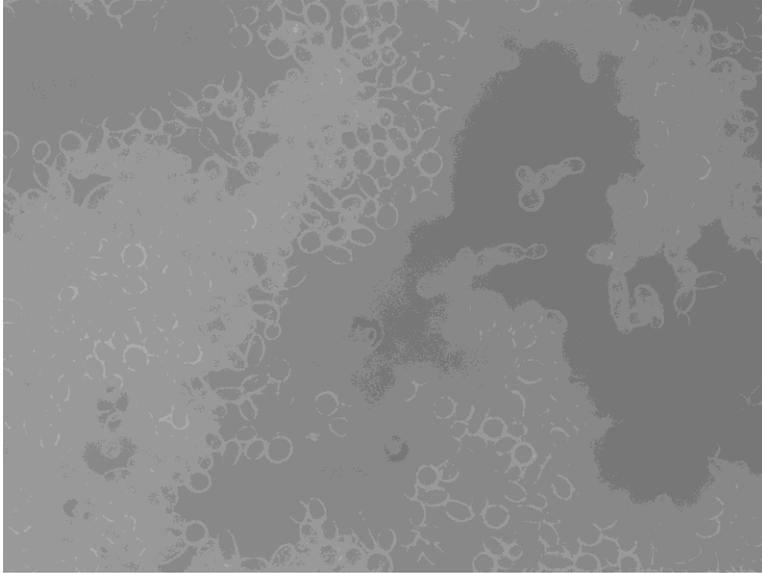


Figure 3. *C. albicans* cells in planktonic form after exposure to 300  $\mu$ M Farnesol for 24 hours and grown on BSA.

### **Research Project 2: Project Title and Purpose**

*Impact of N-Acetyl Cysteine on Heat Shock Protein 70* – The goal of our study is to understand the mechanism of action of N-acetyl cysteine (NAC). NAC has long been used to raise the critical antioxidant glutathione. However, our recent data suggest that it also increases heat shock protein 70, a chaperone that helps reduce the cellular burden of misfolded proteins. This may better explain why NAC has benefited patients with Alzheimer’s disease in clinical trials, because Alzheimer’s disease is primarily a disorder of protein misfolding. If we can show that NAC protects cells by raising heat shock protein 70, this would yield novel insight into its efficacy. Such a finding would usher in a new field of research on the impact of NAC on the heat shock family of proteins, a set of protective chaperone proteins.

### **Anticipated Duration of Project**

1/1/2013 – 12/31/2015

### **Project Overview**

The overarching goal is to understand why N-acetyl cysteine (NAC) protects against animal models of neurodegeneration and improves cognitive function in Alzheimer’s patients. Although NAC is well known to be a glutathione precursor, recent data collected in the Leak laboratory show that NAC also raises the folding chaperone heat shock protein 70 (Hsp70). Furthermore, pilot data suggest that inhibition of Hsp70 activity prevents the protective action of NAC. These novel findings reveal an unanticipated level of complexity to this inexpensive over-the-counter dietary supplement and warrant deeper mechanistic investigations. We propose two Specific

Aims to test the main hypothesis that *NAC protects cells through a rise in Hsp70*. Aim 1: We will test the hypotheses that an adenosine triphosphatase (ATPase) inhibitor of Hsp70 (MAL3-101) and knockdown of Hsp70 messenger Ribonucleic Acid (mRNA) levels with Ribonucleic Acid (RNA) interference will both result in loss of the protective effect of NAC against MG132 toxicity in neuronal N2a cells and primary astrocytes. In order to elicit degeneration, we apply the toxic proteasome inhibitor MG132 to neurons and glia, the two basic cell types in the brain. MG132 raises the levels of misfolded proteins by inhibiting misfolded protein degradation. If our findings generalize across two cell types, this will support the robustness of the effect. Furthermore, using two different means of inhibiting Hsp70 will reduce the likelihood that the results are from non-specific inhibition of other proteins and will help solidify the role of this chaperone. Other heat shock proteins (Hsp90, Hsp40, and Hsp25) will also be measured to test the hypothesis that NAC raises multiple members of this protein family. Aim 2: We will test the hypothesis that NAC raises Hsp70 levels in the brain of animals *in vivo* and that it protects neuronal and glial cells from infusions of MG132 into the rat hippocampus. The hippocampus is involved in learning and memory and is severely affected in Alzheimer's disease. Aim 2 will accomplish 4 goals. First, it examines the impact of NAC in live animals and corroborates our *in vitro* Aim 1. Second, the impact of NAC on Hsp70 can be simultaneously examined in multiple cell types (neurons and glia) and various tissues (brain, liver, and blood) to reveal how well the findings can be generalized. Third, other heat shock proteins will also be assessed to examine the impact of NAC on the heat shock protein family *in vivo*. Fourth, a model of hippocampal loss in Alzheimer's will be developed for general use by the Leak laboratory by infusing MG132 into this vulnerable brain region.

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### **Expected Research Outcomes and Benefits**

This research is expected to shed light on the underlying mechanism of action of N-acetyl cysteine (NAC). NAC benefits Alzheimer's patients' cognitive function and is currently in clinical trials for Parkinson's disease. This grant is an example of "bedside-to-bench" medicine, where drugs known to be useful in the clinic are reexamined in experimental models to better understand how they function. This reverse approach helps to 1) shed light into disease etiology, 2) identify novel therapeutic targets, and 3) design even better drugs with fewer side effects. This approach has already been employed successfully in psychiatry for many decades and it is time it is leveraged against the devastating diseases associated with the aging population of America.

The present study hopes to identify additional, novel targets of NAC other than its well-known impact on glutathione. Our pilot data suggest that heat shock protein 70 (Hsp70) is one such promising target. Although heat shock proteins are protective in animal models of disease, whether Hsp70 protects patients against degeneration is not known. Thus, our long-term goal is to help focus clinical research on this protein. For example, blood levels of Hsp70 before and after an oral NAC regimen could be measured to see if Hsp70 levels correlate with improved human cognition. Furthermore, we hope to examine the breadth of NAC's actions on the entire heat shock protein family. Hsp25, Hsp90, and Hsp40 will be measured in the present study to begin this process. All these studies will benefit patients because they help to identify additional therapeutic targets of a compound already known to be protective. Finally, the studies may directly improve health status by encouraging the use of this inexpensive dietary supplement by those at high risk for disease, such as the elderly with a family history of neurodegeneration.

### **Summary of Research Completed**

*Aim 1: test the hypotheses that an adenosine triphosphatase (ATPase) inhibitor of Hsp70 (MAL3-101) and knockdown of Hsp70 messenger Ribonucleic Acid (mRNA) levels with Ribonucleic Acid (RNA) interference will both result in loss of the protective effect of NAC against MG132 toxicity in neuronal N2a cells and primary astrocytes.*

In the last reporting period (July 2013-July 2014) we measured the impact of NAC on heat shock proteins other than Hsp70, as originally proposed. For these studies, we focused on four essential heat shock proteins, Hsp25, which battles apoptosis, Hsp32, also known as heme oxygenase 1 (HO1), which functions in antioxidant defense, Hsp40, which stimulates Hsp70 activity, and Hsp90, which has client proteins that is folds and stabilizes. Primary glial astrocytes harvested from postnatal cerebral cortex of 1 day-old rat pups were treated with the proteasome inhibitor MG132 (0.2  $\mu$ M) or vehicle (dimethyl sulfoxide) in the presence of NAC (3 mM) or vehicle (sterile water). Cells were harvested 24h later and infrared Western immunoblotting was performed (Fig 1A-D). MG132 elicited an increase in all four proteins, probably as a compensatory reaction to regain homeostatic equilibrium (\* $p \leq 0.01$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  vehicle vs MG132). In the presence of NAC, the impact of MG132 on HO1 and Hsp40 was significantly attenuated (+ $p \leq 0.01$ , ++ $p \leq 0.01$ , +++ $p \leq 0.001$  vehicle vs NAC) whereas the impact of MG132 on Hsp25 was not affected. MG132 also did not lead to a significant rise in Hsp90 in NAC-treated cells. These findings suggest that NAC does not increase these heat shock proteins in cortical astrocytes, unlike in the N2a neuroblastoma model.

In the original grant submission, we had also proposed to examine with two independent techniques whether NAC protects astrocytes in an Hsp70-dependent manner. In the last progress report, we showed that inhibition of Hsp70/Hsc70 activity with VER155008 abolished the protective effects of NAC in cortical astrocytes. Hsc70 is the constitutive form of Hsp70 and exhibits 90% sequence homology to Hsp70. Therefore, Hsp70 and Hsc70 have similar biochemical properties and inhibitors of Hsp70 activity also inhibit Hsc70. In order to validate the VER155008 findings, we have used a second pharmacological inhibitor of Hsp70/Hsc70 activity, 2-phenylethynylsulfonamide (PES). As expected, NAC was protective against MG132 in primary cortical astrocytes and PES abolished its protective effects (Fig. 1E-G). As in the last progress report, the data are expressed in two ways. In Fig 1E, the astrocyte cell counts are

expressed as a percentage of the vehicle-treated astrocytes. MG132 elicited significant cell loss ( $*p \leq 0.01$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$  vehicle vs MG132), but this effect was abolished by NAC ( $+p \leq 0.01$ ,  $++p \leq 0.01$ ,  $+++p \leq 0.001$  vehicle vs NAC). In the presence of PES, there was baseline toxicity ( $\wedge p \leq 0.01$ ,  $\wedge\wedge p \leq 0.01$ ,  $\wedge\wedge\wedge p \leq 0.001$  vehicle vs PES), and MG132 elicited cell loss even in the NAC-treated group when Hsp70/Hsc70 activity was inhibited. In Fig.1F, the astrocyte cell counts from the same experiments as in Fig.1E are expressed as a percentage of each 0  $\mu$ M MG132 group. This allows for the proper statistical comparisons of the effects of PES without the confounding reduction in baseline viability. Fig.1F therefore verifies that the protective effects of NAC against MG132 are indeed attenuated when Hsp70/Hsc70 activity is inhibited with PES. PES and VER155008 work through different mechanisms of action to inhibit Hsp70/Hsc70. The similarities of their effects on NAC-mediated protection therefore strongly suggest that the effects are specific to Hsp70/Hsc70 inhibition. Taken together with the VER155008 from the last report, our data now demonstrate that the protective effects of NAC in astrocytes are indeed mediated by Hsp70/Hsc70 chaperone activity.

In the past year, the lab has moved away from N2a neuroblastoma cells to primary cortical neurons as a superior model for neurodegenerative disorders. Neuroblastoma cells are transformed and still dividing, whereas neurons are post-mitotic. Therefore, we have conducted additional studies on NAC in primary neuron cultures. For these studies, we harvested primary cortical neurons from a highly vulnerable subregion of the cerebral cortex, the temporal lobe. We have focused on this region because the entorhinal and piriform allocortex from the temporal lobe develops pathology in both Alzheimer's and Parkinson's disease. We found that NAC also protects primary allocortical neurons from MG132 toxicity (Fig.1H-J;  $***p \leq 0.001$  vehicle vs MG132;  $+++p \leq 0.001$  vehicle vs NAC). In these studies, the neuronal phenotypic marker microtubule associated protein 2 (MAP2) was used to measure neuronal viability by fluorescent In-Cell Western analyses (Fig.1H, I). MAP2-immunostained neurons were also visualized by higher resolution microscopy in Fig.1J. The quantitative In-Cell Western and qualitative microscopy data are in agreement that NAC significantly protects allocortical neurons.

Next we tested the hypothesis that the protective effects of NAC in primary neurons are abolished when Hsp70/Hsc70 activity is inhibited. In support of this hypothesis, we found that VER155008 greatly increased the toxicity of MG132 in all groups and that the protective effects of NAC (375  $\mu$ M) were completely abolished (Fig.1K,L  $*p \leq 0.01$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$  vehicle vs MG132;  $++p \leq 0.01$  vehicle vs NAC;  $\wedge\wedge p \leq 0.001$  vehicle vs VER155008). These findings suggest that NAC-treated primary allocortical neurons use Hsp70/Hsc70 to defend themselves against MG132 toxicity and are entirely consistent with the findings in N2a neuroblastoma cells and primary astrocytes. Our recent studies therefore show that the Hsp70/Hsc70-dependent protective effects of NAC can be generalized across many different cell types.

*Aim 2: test the hypothesis that NAC raises Hsp70 levels in the brain of animals in vivo and that it protects neuronal and glial cells from infusions of MG132 into the rat hippocampus.*

For the second Aim of these studies, we have initiated *in vivo* experiments. We are still in the process of developing an MG132-treated animal model of Alzheimer's disease and a 6-hydroxydopamine (6-OHDA)-treated animal model of Parkinson's disease in which to test the protective effects of NAC. We have come across some issues with MG132 dissolution in saline.

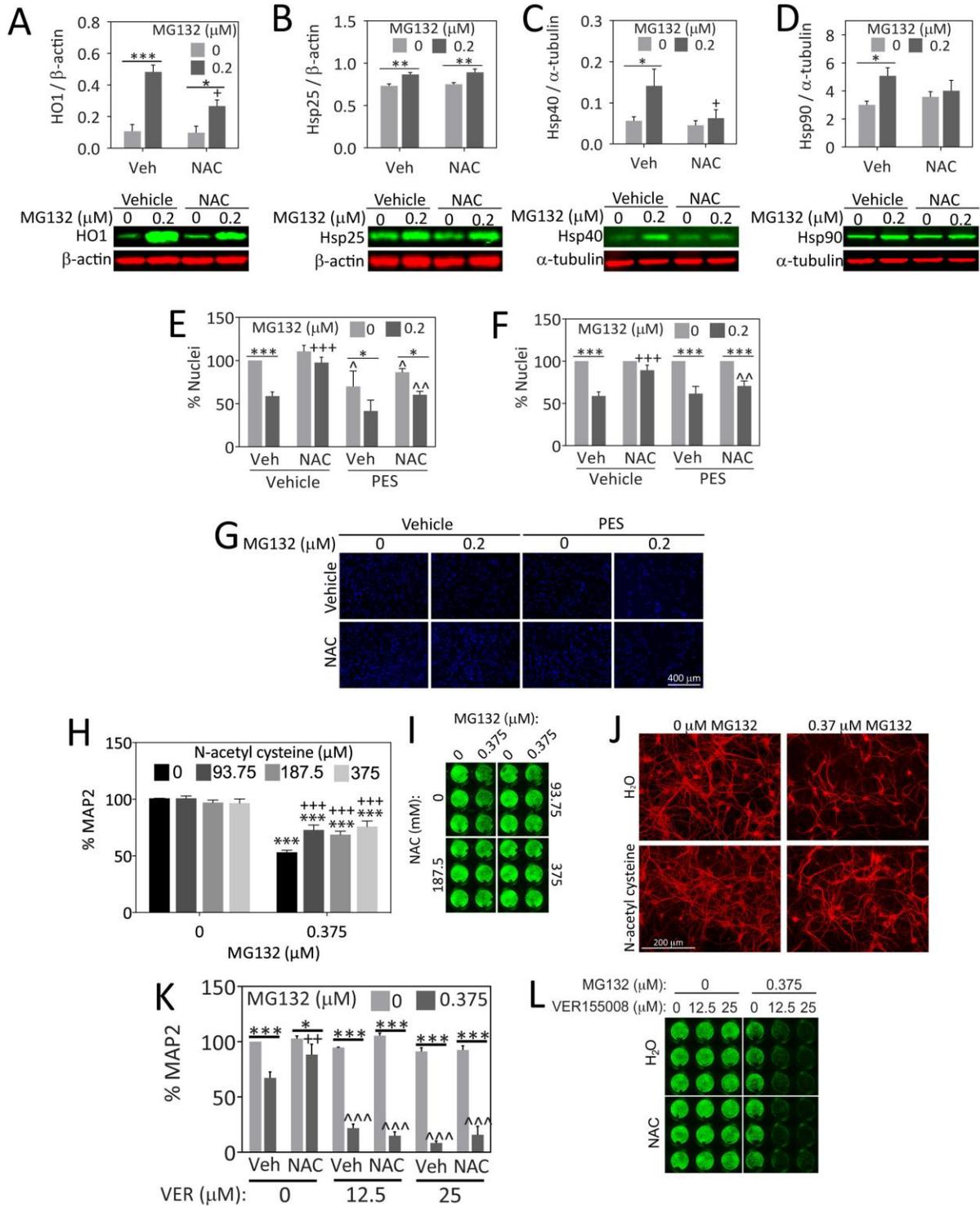
MG132 dissolves more readily in dimethyl sulfoxide (DMSO). However, injecting MG132 in DMSO is not ideal, as this vehicle can be toxic when infused into the brain. We are currently trying to dissolve MG132 in small volumes of DMSO, followed by further dilution in saline.

As we have made more progress with 6-OHDA, we will describe those findings here. 6-OHDA is an oxidative toxin that is taken up by dopamine neurons upon infusion into the striatum. Dopamine neurons are then killed by the oxidative stress of 6-OHDA and loss of dopamine neurons elicits the parkinsonian motor deficits. We began this study with a 6-OHDA dose-response curve. Mice were unilaterally infused with 6-OHDA into the striatum and sacrificed by perfusion one week later. Striatal sections were immunostained for the dopaminergic phenotypic marker tyrosine hydroxylase (TH). TH is expressed in dopamine terminals in this brain region and is greatly reduced in Parkinson's disease. In these studies, TH immunostaining in the infused striatum was expressed as a percentage of TH levels in the contralateral, control hemisphere (Fig.2A). As expected, 6-OHDA elicited dose-responsive loss of dopamine terminals in the ipsilateral striatum ( $*p\leq 0.01$ ,  $**p\leq 0.01$ ,  $***p\leq 0.001$  vehicle vs 6-OHDA). For example, the 4  $\mu\text{g}$  6-OHDA dose elicited approximately 40% loss of TH. These findings show that we have established a reliable model of the dopaminergic loss in Parkinson's disease.

Next we proceeded to test the hypothesis that NAC would protect against striatal TH loss. We infused mice with 4  $\mu\text{g}$  6-OHDA or vehicle into the striatum, followed by daily intraperitoneal delivery of NAC (100 mg/kg) or vehicle (saline). One week later, animals were sacrificed and analyzed for TH immunostaining in the striatum. NAC significantly raised the amount of TH remaining after 6-OHDA infusion (Fig.2B;  $\sim p\leq 0.01$ , vehicle vs NAC). However, this statistical change was entirely attributable to an increase in baseline TH immunoreactivity, as there was a trend towards the same effect in the contralateral, untreated hemisphere ( $p=0.058$  vehicle vs NAC). In order to show more convincingly that there was no true protection, we also expressed the TH staining in the ipsilateral hemisphere as a percentage of the staining in the contralateral hemisphere (Fig.2C). These data reveal that NAC does not modify the percentage loss of TH after 6-OHDA infusion. In other words, 6-OHDA was equally toxic in both saline and NAC-treated groups ( $**p\leq 0.01$ ,  $***p\leq 0.001$  vehicle vs 6-OHDA). These results show that NAC does not really protect dopamine neurons in our model of Parkinson's disease. However, the animals may have been stressed by the daily intraperitoneal injections that lasted an entire week and this may have attenuated any protective effects. We are therefore in the process of repeating these experiments with NAC delivered more unobtrusively in drinking water. If we can establish a protective effect of NAC *in vivo*, we will also measure heat shock protein levels in the striatum.

**Statistics:** For all the *in vitro* experiments described above, we conducted at least 3 independent experiments, each run in triplicate wells. The *in vitro* data were analyzed with the Bonferroni *post hoc* correction following a two-way or three-way ANOVA (SPSS, Version 20). For all the *in vivo* experiments, 4-13 mice were treated for each group and the data were analyzed with the Bonferroni *post hoc* correction following a one-way or two-way ANOVA. Data were deemed significant only when  $p\leq 0.05$ .

**Figure 1**



**Figure 2**

