Published in final edited form as:

Curr Neurovasc Res. 2009 February ; 6(1): 20–31.

The Forkhead Transcription Factor FOXO3a Controls Microglial Inflammatory Activation and Eventual Apoptotic Injury through Caspase 3

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Abstract

Memory loss and cognitive failure are increasingly being identified as potential risks with the recognized increase in life expectancy of the general population. As a result, the development of novel therapeutic strategies for disorders such as Alzheimer's disease have garnered increased attention. The etiologies that can lead to Alzheimer's disease are extremely varied, but a number of therapeutic options are directed against amyloid- β peptide and inflammatory cell regulation to prevent or halt progressive cognitive loss. In particular, inflammatory microglial cells may have disparate functions that in some scenarios lead to disability through the removal of functional neurovascular cells and in other circumstances foster tissue repair. Given the significance microglial cells hold for neurodegenerative disorders, we therefore examined the function that amyloid ($A\beta_{1-42}$) has upon the microglial cell line EOC 2 and identified a novel role for the forkhead transcription factor FoxO3a and caspase 3. Here we show that $A\beta_{1-42}$ leads to progressive injury and apoptotic cell loss in microglial cells that involves both early phosphatidylserine (PS) externalization and late genomic DNA fragmentation over a 24 hour course. Prior to these injury programs, $A\beta_{1-42}$ results in the activation and proliferation of microglia as demonstrated by increased proliferating cell nuclear antigen (PCNA) expression and bromodeoxyuridine (BrdU) uptake. Both apoptotic injury as well as the prior activation and proliferation of microglial cells relies upon the presence of FoxO3a, since specific gene silencing of FoxO3a promotes microglial cell protection and prevents the early activation and proliferation of these cells. Furthermore, $A\beta_{1-42}$ exposure maintained FoxO3a in an unphosphorylated "active" state and facilitated the cellular trafficking of FoxO3a from the cytoplasm to the cell nucleus to potentially lead to "pro-apoptotic" programs by this transcription factor. One apoptotic program in particular appears to involve the activation of caspase 3, since loss of FoxO3a through gene silencing prevents the induction of caspase 3 activity by $A\beta_{1-42}$.

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Keywords

Alzheimer's disease; amyloid; apoptosis; bromodeoxyuridine; caspases; forkhead transcription factors; FoxO3a; inflammation; microglia; oxidative stress; proliferating cell nuclear antigen

INTRODUCTION

With enhanced life expectancy in the general population, the possibility of memory loss and impaired cognition that leads to progressive disability is becoming an increasing concern for individuals (Chong, *et al.*, 2005d, Maiese and Chong, 2004). In this regard, disorders such as Alzheimer's disease have attracted increased attention for the development of novel therapeutic strategies. The etiologies that can lead to Alzheimer's disease are broad in nature (Chong, *et al.*, 2005c) and can include therapies directed through chemokine receptors (Bakshi, *et al.*, 2008), calpain inhibition (Vaisid, *et al.*, 2008), cell cycle regulation (Chong, *et al.*, 2006a, Lin, *et al.*, 2001, Maiese, *et al.*, 2008d, Majd, *et al.*, 2008), presenilins (Maiese, *et al.*, 2008b, Newman, *et al.*, 2007), protein kinase B (Chong, *et al.*, 2004a, Chong, *et al.*, 2002, Damjanac, *et al.*, 2008, Kang, *et al.*, 2003), oxidative stress (De Felice, *et al.*, 2007, Maiese, *et al.*, 2009a, Maiese, *et al.*, 2008a, Power, *et al.*, 2008), and growth factor activation (Chong, *et al.*, 2005b, Maiese, *et al.*, 2008a, Maiese, *et al.*, 2005, Sun, *et al.*, 2008).

Yet, many treatment options for Alzheimer's disease are directed against amyloid- β peptide and inflammatory cell regulation to reduce or eliminate injury to cognitive centers of the brain. Accumulation of A β_{1-42} can lead to oxidative stress, cytokine alterations, apoptotic injury, and to a significant inflammatory response with microglial activation (Chong, *et al.*, 2007a, Chong, *et al.*, 2005b, Kawamoto, *et al.*, 2008). Interestingly in regards to microglial activation, microglia in the nervous system can lead to the phagocytic removal of both neurons and vascular cells (Chong, *et al.*, 2005a, Chong, *et al.*, 2004a, Kang, *et al.*, 2003). Although microglia may lead to cell injury (Bureau, *et al.*, 2008, Chong, *et al.*, 2005a, Chong, *et al.*, 2007a) and have been tied to Alzheimer's disease (Sheng, *et al.*, 1997), microglia also require cytoprotective pathways (Chong, *et al.*, 2007b, Li, *et al.*, 2006) to proliferate, remove injured cells (Li, *et al.*, 2005, Mallat, *et al.*, 2005), and promote tissue repair (Chong, *et al.*, 2007b, Dringen, 2005).

In light of the impact microglial cells hold upon degenerative disorders such as Alzheimer's disease, we examined the function that amyloid (A β_{1-42}) has upon these cells as well as the novel role of the forkhead transcription factor FoxO3a. Mammalian forkhead transcription factors of the O class (FoxOs) that include FoxO3a have emerged as important modulators of cell apoptosis and immune function (Maiese, *et al.*, 2008c, Maiese, *et al.*, 2007, Maiese, *et al.*, 2008d, Maiese, *et al.*, 2009b). For example, mice that are deficient for FoxO3a have increased activity of helper T cells results, supporting an important role for FoxO3a in preventing T cell hyperactivity (Lin, *et al.*, 2004). FoxO3a also appears to be necessary for neutrophil activity, since FoxO3a null mice are resistant to models of neutrophilic inflammation (Jonsson, *et al.*, 2005). Furthermore, pathways that involve caspase activity may be linked to the forkhead transcription factor FoxO3a. Increased activity of FoxO3a can result in cytochrome c release and caspase-induced apoptotic death (Chong, *et al.*, 2006b, Chong, *et al.*, 2004c, Chong and Maiese, 2007a, Obexer, *et al.*, 2007). In addition, blockade of caspase 3 has been shown to maintain the phosphorylated "inactive" state of FoxO3a to preserve cellular survival (Chong, *et al.*, 2006b, Chong, *et al.*, 2004c, Chong and Maiese, 2007a). Pathways with caspase 3 may be vital for microglial cell regulation since caspase 3 has been associated with the apoptotic pathways of genomic DNA cleavage, cellular membrane PS exposure, and activation of inflammatory cells (Chong, *et al.*, 2003a, Chong, *et al.*, 2003b, Chong, *et al.*, 2004b). Here we demonstrate that A β_{1-42} exposure leads to initial caspase 3 activity with 6

hours and to subsequent apoptotic injury in microglial cells. However, prior to cell injury, microglial cells are activated by A β _{1–42} exposure and begin cell programs consistent with proliferation. Through gene deletion studies, we show that both early and late programs for microglial apoptotic injury as well as the prior phase of microglial activation and proliferation during A β _{1–42} exposure can be averted through the specific knockdown of FoxO3a that controls caspase 3 activation.

MATERIALS AND METHODS

Microglia Cell Cultures

The microglial cell line EOC 2 was obtained from American Type Culture Collection (ATTC, Manassas, VA.). Cells were maintained in Dulbecco's modified Eagle medium (ATTC, Manassas, VA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO), 50 μ g/ml penicillin and streptomycin and 20% media from the LADMAC cell line (ATCC, Manassas, VA) which contains colony stimulating factor-1 (CSF-1) secreted by LADMAC cells. Cells were seeded onto 24-well plates or 35 mm culture dishes at a density of 1.5×10^6 cells per well or 4×10^6 cells per dish.

Experimental Treatments

β -Amyloid (A β _{1–42}) Treatment—A β _{1–42} (US Peptide, Rancho Cucamonga, CA) was dissolved in PBS at a concentration of 200 μ M. To allow for A β aggregation, A β was incubated at 37°C for a 7 day period and then directly applied to microglial cell cultures at final concentrations per the experimental protocols.

Assessment of Cell Survival

Microglial injury was determined by bright field microscopy using a 0.4% trypan blue dye exclusion method 24 hours following treatment with A β _{1–42} per our previous protocols (Chong, *et al.*, 2005b). The mean survival was determined by counting eight randomly selected non-overlapping fields with each containing approximately 10–20 cells (viable + non-viable). Each experiment was replicated 6 times independently with different cultures.

Assessment of DNA Fragmentation

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay (Chong, *et al.*, 2002, Kang, *et al.*, 2003). Briefly, microglial cells were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde and the 3'-hydroxy ends of cut DNA were labeled with biotinylated dUTP using the enzyme terminal deoxytransferase (Promega, Madison, WI) followed by streptavidin-peroxidase and visualized with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA).

Assessment of Membrane Phosphatidylserine (PS) Residue Externalization

Phosphatidylserine (PS) exposure was assessed through the established use of annexin V. Per our prior protocols (Chong, *et al.*, 2004a, Kang, *et al.*, 2003), a 30 μ g/ml stock solution of annexin V conjugated to phycoerythrin (PE) (R&D Systems, Minneapolis, MN) was diluted to 3 μ g/ml in warmed calcium containing binding buffer (10 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂). Plates were incubated with 500 μ l of diluted annexin V for 10 minutes. Images were acquired with "blinded" assessment with a Leitz DMIRB microscope (Leica, McHenry, IL) and a Fuji/Nikon Super CCD (6.1 megapixels) using transmitted light and fluorescent single excitation light at 490 nm and detected emission at 585 nm.

Assessment of Microglial Activation and Proliferation

Proliferating cell nuclear antigen (PCNA) expression for microglial activation (Williams, *et al.*, 2002) and bromodeoxyuridine (BrdU) uptake for microglial proliferation (Martinez-Contreras, *et al.*, 2002) was performed with anti-mouse monoclonal antibody PCNA (1:1000) or BrdU (1:6000) (Sigma, St Louis, MO) conjugated with biotinylated anti-mouse IgG (1:100) and visualized through fluorescein avidin (1:100) for PCNA and Texas Red streptavidin (Vector laboratories, Burlingame, CA) for BrdU. BrdU (10 μ M) and fluorodeoxyuridine (1 μ M) (Sigma, St. Louis, MO) were applied 1 hour prior to the time of fixation.

Expression of Phosphorylated FoxO3a, total FoxO3a, and Active Caspase 3

Cells were homogenized and each sample (50 μ g lane⁻¹) was subjected to 7.5% SDS-polyacrylamide gel electrophoresis. After transfer, the membranes were incubated with a rabbit polyclonal antibody against a rabbit antibody against phospho-FoxO3a (1:1000) (p-FOXO3a, Ser²⁵³, Cell Signaling, Beverly, MA), a rabbit antibody against total FoxO3a, and a rabbit antibody against cleaved (active) caspase 3 (Cell Signaling, Beverly, MA). Following washing, the membranes were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody goat anti-rabbit IgG (1:2000, Zymed Laboratories, Carlsbad, CA). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and band density was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>).

Gene Silencing of FoxO3a with Small Interfering RNA (siRNA)

To silence FOXO3a gene expression, the following sequences were synthesized (Ambion, Austin, Texas): the FOXO3a target sequence 5'-AAATCTAACTCATCTGCA AGT -3', the siRNA sense strand 5'-AUCUAAACUCAUC UGCAAGUUU -3', and the antisense strand 5'-ACUUGCA GAUG AGUUAGAUAUUU -3'. Transfection of siRNA duplexes were performed with Lipofectamine 2000 reagent according to manufacturer guidelines (Invitrogen, Carlsbad, CA). Experimental assays were performed 72 hours post-transfection. For each siRNA assay, positive controls contain multiple siRNAs including the target siRNA and negative controls are absent of the target siRNA.

Immunocytochemistry for FoxO3a

For immunocytochemical staining of FoxO3a, microglial cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100. Cells were then incubated with rabbit anti-FoxO3a (1:100, Cell Signaling Technology, Beverly, MA) over night at 4°C and then with biotinylated anti-rabbit IgG (1:50, Vector laboratories) for 2 hours followed by Texas Red streptavidin (1:50, Vector laboratories) for 1 hour. Cells are washed in PBS, then stained with DAPI (Sigma, St. Louis, MO) for nuclear identification. FoxO3a protein was imaged with fluorescence at the wavelengths of 565 nm (red) and 400 nm (DAPI nuclear staining).

Statistical Analysis

For each experiment, the mean and standard error were determined. Statistical differences between groups were assessed by means of analysis of variance (ANOVA) from 6 replicate experiments with the post-hoc Dunnett's test. Statistical significance was considered at $p < 0.05$.

RESULTS

Amyloid (A β ₁₋₄₂) is toxic to microglial cells and leads to apoptotic genomic DNA degradation and membrane phosphatidylserine (PS) exposure.

A β_{1-42} was applied to microglia in a series of concentration (1, 5, 10, and 20 μ M) and cell survival was assessed 24 hours later by trypan blue dye exclusion. As shown in Fig. (1A top panel), A β_{1-42} (10 μ M) lead to increased trypan blue uptake in cells. In Fig. (1A bottom panel), microglial survival was significantly reduced to $73 \pm 2.9\%$, $41 \pm 3.4\%$, and $19 \pm 4.3\%$ at the concentrations of 5 μ M, 10 μ M, and 20 μ M 24 hours following A β_{1-42} administration when compared to untreated control cells with a survival of $91.1 \pm 2.3\%$, $p < 0.01$. The A β_{1-42} concentration of 1 μ M was not significantly toxic with a cell survival of $84 \pm 3.2\%$. Since the A β_{1-42} concentration of 10 μ M provided a robust cell injury model and resulted in a survival of approximately 40% (60% microglial cell loss), this concentration of A β_{1-42} was employed for the subsequent experimental paradigms.

Apoptosis consists of both late genomic DNA degradation as well as early membrane PS exposure (Chong, *et al.*, 2005c). In addition, the exposure of membrane PS residues in cells has been tied to inflammatory cell activation, phagocytosis (Chong, *et al.*, 2005a, Maiese, *et al.*, 2005, Simak, *et al.*, 2002), and the identification of cells infected with virions (Soares, *et al.*, 2008) that may extend cell survival of microglial cells (Chugh, *et al.*, 2007). We therefore examined the effects of A β_{1-42} (10 μ M) upon DNA degradation and PS exposure. In Fig. (1B top panel), microglial cells were exposed to A β_{1-42} and 24 hours later cell apoptosis was assessed by DNA fragmentation using TUNEL and by cell membrane PS exposure determined by annexin V. Untreated control cells were without DNA fragmentation or PS externalization. In cells exposed to A β_{1-42} (10 μ M) there was significant DNA fragmentation and membrane PS exposure. Quantification of results in Fig. (1B bottom panel) illustrated an increase in DNA fragmentation ($56 \pm 5\%$) and in membrane PS exposure ($58 \pm 5\%$) at 24 hours after exposure to A β_{1-42} when compared to untreated control cultures (DNA fragmentation, $8 \pm 3\%$; PS exposure, $11 \pm 3\%$).

Amyloid (A β_{1-42}) Leads to Early Microglial Activation and Proliferation

Since activation of microglial cells can lead to inflammation and the death of a number of cell types such as in the nervous system (Bureau, *et al.*, 2008, Chong, *et al.*, 2005a, Chong, *et al.*, 2007a), we next investigated whether A β_{1-42} altered microglial activation and proliferation. In Fig. (2A), representative microglial cultures illustrate a marked induction of microglial activation and proliferation following treatment with A β_{1-42} (10 μ M) at 3 hours and 6 hours as evidenced by increased PCNA expression (Williams, *et al.*, 2002) and BrdU uptake (Martinez-Contreras, *et al.*, 2002) when compared to untreated microglia treated (control). In Fig. (2B), quantification of PCNA and BrdU labeling revealed that a significant expression in PCNA at 3 hours ($31 \pm 6\%$, $p < 0.01$) and 6 hours ($68 \pm 4\%$, $p < 0.01$) as well as BrdU uptake at 3 hours ($36 \pm 6\%$, $p < 0.01$) and 6 hours ($73 \pm 6\%$, $p < 0.01$) was present in microglia cultures following the application of A β_{1-42} (10 μ M) when compared to untreated control cultures ($13 \pm 4\%$, PCNA; $17 \pm 5\%$, BrdU).

Amyloid (A β_{1-42}) Results in the Loss of FoxO3a Phosphorylation and Translocation of FoxO3a to the Cell Nucleus

Western blot assay was performed for phosphorylated FoxO3a (p-FoxO3a) at the preferential phosphorylation site for Akt of Ser²⁵³ as well as for the expression of total FoxO3a at 6 hours following A β_{1-42} (10 μ M) exposure (Figs. 3A, 3B). After 6 hours post A β_{1-42} exposure, expression of p-FoxO3a was lost but expression of total FoxO3a remained unchanged, suggesting that unphosphorylated and active FoxO3a was present rather than the loss of FoxO3a protein through degradation (Figs. 3A, 3B).

Since active FoxO3a protein that is not phosphorylated can translocate from the cytoplasm to the nucleus to begin a "pro-apoptotic program (Chong and Maiese, 2007a, Maiese, *et al.*, 2008c, Maiese, *et al.*, 2008d, Maiese, *et al.*, 2009b), we next performed immunofluorescent

staining for FoxO3a and DAPI nuclear staining to follow the subcellular translocation of FoxO3a in microglia 6 hours following A β ₁₋₄₂ (10 μ M) exposure (Figs. 3C, 3D). During A β ₁₋₄₂ administration, significant immunofluorescent staining for FoxO3a in the nucleus of microglia is present. This is evident by the inability to detect significant DAPI nuclear staining (blue in color) in cells during merged A β ₁₋₄₂ images since prominent FoxO3a staining is present in the nucleus (Figs. 3C, 3D). In contrast, without A β ₁₋₄₂ exposure (control), FoxO3a is retained in the cytoplasm with minimal nuclear staining as shown with the notable presence of DAPI nuclear staining (white in color) in merged images. In Fig. (3D), data analysis demonstrates that microglial nuclear staining area was significantly increased ($167 \pm 5\%$) 24 hours following a 6 hour period of A β ₁₋₄₂ administration when compared to untreated control cells.

Gene Silencing of FoxO3a Increases Microglial Cell Survival and Prevents Apoptotic DNA Fragmentation and Phosphatidylserine (PS) Exposure

Microglial cells were transfected with FoxO3a siRNA and the expression of total FoxO3a protein was observed through Western blot analysis (Figs. 4A and 4B). Gene silencing of FoxO3a in untreated control cells or during A β ₁₋₄₂ exposure (10 μ M) significantly reduced or eliminated the expression of total FoxO3a 6 hours after A β ₁₋₄₂ exposure (Figs. 4A and 4B). In Fig. (4C), representative figures illustrate significant trypan blue staining in microglia during A β ₁₋₄₂ exposure (10 μ M) alone or with A β ₁₋₄₂ exposure (10 μ M) during scrambled (non-specific) siRNA 24 hours after A β ₁₋₄₂ exposure. However, significantly less trypan blue uptake is present in cell exposed to A β ₁₋₄₂ (10 μ M) with FoxO3a siRNA transfection for 3 days. During transfection of microglia with FoxO3a siRNA (Fig. 4D), microglial survival was increased from $43 \pm 5\%$ during A β ₁₋₄₂ (10 μ M) alone to $62 \pm 4\%$ during A β ₁₋₄₂ with FoxO3a siRNA 24 hours after A β ₁₋₄₂ exposure, illustrating that elimination of FoxO3a can enhance microglial survival during A β ₁₋₄₂ exposure. Transfection with scrambled siRNA did not protect microglial cells during A β ₁₋₄₂ administration.

We next examined the ability of gene silencing of FoxO3a to alter apoptotic genomic DNA fragmentation and membrane PS exposure in microglia following A β ₁₋₄₂ (10 μ M) exposure. In Fig. (5A), representative figures show a marked decrease in DNA fragmentation as assessed by TUNEL and PS exposure as assessed by annexin V during A β ₁₋₄₂ exposure (10 μ M) in cells transfected with FoxO3a siRNA, but no apoptotic protection in microglia transfected with scrambled siRNA. In Fig. (5B), transfection of microglia with FoxO3a siRNA decreased DNA fragmentation from $61 \pm 5\%$ during A β ₁₋₄₂ (10 μ M) exposure alone to $35 \pm 2\%$ during A β ₁₋₄₂ with FoxO3a siRNA and decreased apoptotic PS exposure from $58 \pm 5\%$ during A β ₁₋₄₂ (10 μ M) alone to $31 \pm 4\%$ during A β ₁₋₄₂ with FoxO3a siRNA, demonstrating that loss of FoxO3a leads to protection against apoptotic DNA fragmentation and membrane PS exposure.

Removal of FoxO3a through Gene Silencing Blocks Increases Microglial Cell Activation and Proliferation

In Fig. (6A), representative microglial cultures show a significant reduction in the activation (PCNA expression) and proliferation (BrdU uptake) of microglia at 6 hours following administration of A β ₁₋₄₂ (10 μ M) when compared to microglia treated with A β ₁₋₄₂ alone. In addition, transfection of cells with scrambled siRNA during A β ₁₋₄₂ exposure did not alter PCNA expression or BrdU uptake when compared with cells treated with A β ₁₋₄₂ alone. Fig. (6B) demonstrates that transfection of microglia with FoxO3a siRNA 6 hours following A β ₁₋₄₂ (10 μ M) exposure decreased PCNA expression from $61 \pm 4\%$ during A β ₁₋₄₂ exposure alone to $35 \pm 5\%$ during A β ₁₋₄₂ with FoxO3a siRNA and decreased BrdU uptake from $66 \pm 5\%$ during A β ₁₋₄₂ alone to $29 \pm 4\%$ during A β ₁₋₄₂ with FoxO3a siRNA. Scrambled siRNA transfection was ineffective in preventing PCNA expression and BrdU uptake during A β ₁₋₄₂

exposure and further supported that gene silencing of FoxO3a was able to prevent microglial activation and proliferation during exposure to A β ₁₋₄₂.

FoxO3a Modulates Caspase 3 Activity during Amyloid (A β ₁₋₄₂) Exposure

Since amyloid may lead to the induction of caspase mediated pathways (Nakagawa, *et al.*, 2000, Troy, *et al.*, 2001) that are involved with oxidative stress (Maiese, *et al.*, 2008b), we examined whether FoxO3a could alter caspase 3 activation in microglia during A β ₁₋₄₂ (10 μ M) exposure. Caspase 3 activity was determined through western analysis of the cleaved caspase 3 product. In Fig. (7A), western blot assay was performed for cleaved caspase 3 at 6 hours following A β ₁₋₄₂ (10 μ M) exposure. After 6 hours post A β ₁₋₄₂ exposure, a significant increase of caspase 3 activity was present with A β ₁₋₄₂ (10 μ M) exposure alone as illustrated with western analysis and percent fold changes compared to untreated microglial cells (control) (Figs. 7A, 7B). Yet, transfection with FoxO3a siRNA markedly prevented caspase 3 activation 6 hours following A β ₁₋₄₂ exposure while transfection of scrambled siRNA was ineffective to prevent caspase 3 activation, demonstrating that FoxO3a is at least one vital component for activation of caspase 3 in microglial cells during A β ₁₋₄₂ exposure (Figs. 7A, 7B).

DISCUSSION

A β ₁₋₄₂ deposition in the brain during Alzheimer's disease is believed to be at least one important component that can promote eventual cognitive loss and clinical decline in individuals. Closely tied to the accumulation of A β ₁₋₄₂ and loss of nervous system function may be the activity of inflammatory microglial cells (Maiese, 2008a, Maiese, 2008b, Maiese, *et al.*, 2008e). We initially show that increases in low concentrations of A β ₁₋₄₂ leads to a progressive loss of microglial cell survival over a 24-hour course. This injury over the 24 hour period in microglia extends to both early and late programs of apoptosis. Apoptotic cell death is considered to be a dynamic process that involves both early membrane phosphatidylserine (PS) externalization (Kang, *et al.*, 2003, Maiese and Chong, 2003) and the later cleavage of genomic DNA into fragments (Chong, *et al.*, 2005c, Chong and Maiese, 2007b). These results are an important consideration since they shed light upon the pathways for the demise of microglia in the nervous system during chronic disorders. Loss of microglia under some conditions may be detrimental, since these cells can be beneficial and form a barrier for the removal of foreign microorganisms and promote tissue repair during cell injury (Maiese, 2008a, Maiese, *et al.*, 2008b).

Interestingly, prior to apoptotic cell pathway induction, microglial cells within the first 3 and 6 hours following A β ₁₋₄₂ exposure become activated and begin to proliferate as evidenced by significant increases in PCNA expression and BrdU uptake. These observations suggest that during this early period of A β ₁₋₄₂ exposure, microglial cells are functional which may represent a double-edge sword. On one side, these activated and proliferating microglia may assisting with tissue repair (Maiese, 2008a, Maiese, *et al.*, 2008b) as well as with novel strategies directed against cancer (Wu, *et al.*, 2008). Yet, on the converse side, activated microglial cells can lead to the phagocytic removal of both neurons and vascular cells (Chong, *et al.*, 2005a, Chong, *et al.*, 2004a, Kang, *et al.*, 2003). Critical loss of these functional neurovascular cells may result in further disability. In addition, microglia also may lead to cellular damage through oxidative stress and through the production of cytokines (Chong, *et al.*, 2005c, Chong, *et al.*, 2005d).

Our work further suggests that the forkhead transcription factor FoxO3a may function as a central component for microglia to not only control inflammatory activation and proliferation, but also to lead to the ultimate demise of microglial cells. In previous animal studies, *Foxo3a* gene loss lead to increased activity of helper T cells (Lin, *et al.*, 2004) and *Foxo3a* null mice were found to be resistant to models of neutrophilic inflammation (Jonsson, *et al.*, 2005), suggesting that FoxO3a can modulate immune system cells. Furthermore, control of

inflammatory activation and apoptosis in the nervous system such as in systemic lupus erythematosus in animal models may require the up-regulation of FoxO3a that can block nuclear factor- κ B (NF- κ B) activation and interferon-gamma secretion (Sela, *et al.*, 2006). In clinical studies, patients with rheumatoid arthritis and osteoarthritis have phosphorylation of FOXO3a in T lymphocytes in synovial macrophages, suggesting that loss of functional FOXO3a also may play a role in controlling inflammatory cell function during rheumatoid arthritis and osteoarthritis (Ludikhuize, *et al.*, 2007). We show that A β ₁₋₄₂ exposure may lead to enhanced activity of FoxO3a by maintaining FoxO3a in an unphosphorylated “active” state. Furthermore, in the presence of A β ₁₋₄₂, we demonstrate that the transcription factor FoxO3a is translocated from the cytoplasm to the cell nucleus of microglia which may govern the initial activation and proliferation of microglia as well as the eventual induction of “pro-apoptotic” programs in these cells (Maiese, *et al.*, 2008c, Maiese, *et al.*, 2007, Maiese, *et al.*, 2008d, Maiese, *et al.*, 2009b). Our subsequent studies support this premise. Through the use of specific gene silencing, a technique that may have eventual utility for the treatment of Alzheimer’s disease (Orlacchio, *et al.*, 2007), we show that transfection of FoxO3a siRNA in microglial cells during A β ₁₋₄₂ exposure increases cell survival and prevents the onset of apoptotic PS externalization and subsequent genomic DNA degradation. Furthermore, FoxO3a appears vital for the activation and cellular proliferation of microglial during A β ₁₋₄₂ exposure since gene silencing of FoxO3a down regulates PCNA expression and BrdU uptake in these cells, suggesting that without FoxO3a microglia are incapable of activation and proliferation.

The cell death pathways for microglia that rely upon FoxO3a also appear to involve caspase 3 activation. Prior studies suggest that not only does FoxO3a activity promote caspase-induced apoptotic death (Chong, *et al.*, 2006b, Chong, *et al.*, 2004c, Chong and Maiese, 2007a, Obexer, *et al.*, 2007), but also demonstrate that inhibition of caspase 3 has been shown to maintain the phosphorylated “inactive” state of FoxO3a to prevent cell injury (Chong, *et al.*, 2006b, Chong, *et al.*, 2004c, Chong and Maiese, 2007a). Other work has shown that caspase 3 activity and cleavage is promoted during transfection of a triple mutant FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of FoxO3a (Gomez-Gutierrez, *et al.*, 2006). Our current work adds further insight to these studies by illustrating that FoxO3a may control apoptotic injury in microglia during A β ₁₋₄₂ exposure through caspase 3. Since A β ₁₋₄₂ exposure facilitates the cellular trafficking of FoxO3a from the cytoplasm to the cell nucleus to potentially lead to “pro-apoptotic” programs by this transcription factor, one program in particular that may be vital for apoptotic injury appears to involve the activation of caspase 3. We illustrate that A β ₁₋₄₂ exposure leads to a rapid and significant increases in caspase 3 activity with 6 hours following A β ₁₋₄₂ administration, but that this induction of caspase 3 activity by A β ₁₋₄₂ requires FoxO3a, since loss of FoxO3a through gene silencing prevents the induction of caspase 3 activity by A β ₁₋₄₂.

Acknowledgements

This research was supported by the following grants (KM): American Diabetes Association, American Heart Association (National), Bugher Foundation Award, Janssen Neuroscience Award, LEARN Foundation Award, MI Life Sciences Challenge Award, Nelson Foundation Award, NIH NIEHS (P30 ES06639), and NIH NINDS/NIA.

References

- Bakshi P, Margenthaler E, Laporte V, Crawford F, Mullan M. Novel role of CXCR2 in regulation of gamma-secretase activity. *ACS Chem Biol* 2008;3:777–89. [PubMed: 19067586]
- Bureau G, Longpre F, Martinoli MG. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. *J Neurosci Res* 2008;86:403–10. [PubMed: 17929310]

- Chong ZZ, Kang J, Li F, Maiese K. mGluRI targets microglial activation and selectively prevents neuronal cell engulfment through Akt and caspase dependent pathways. *Curr Neurovasc Res* 2005a; 2:197–211. [PubMed: 16181114]
- Chong ZZ, Kang JQ, Maiese K. Akt1 drives endothelial cell membrane asymmetry and microglial activation through Bcl-x(L) and caspase 1, 3, and 9. *Exp Cell Res* 2004a;296:196–207. [PubMed: 15149850]
- Chong ZZ, Kang JQ, Maiese K. Apaf-1, Bcl-xL, cytochrome c, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin. *J Cereb Blood Flow Metab* 2003a;23:320–30. [PubMed: 12621307]
- Chong ZZ, Kang JQ, Maiese K. Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways. *Br J Pharmacol* 2003b; 138:1107–1118. [PubMed: 12684267]
- Chong ZZ, Kang JQ, Maiese K. Erythropoietin is a novel vascular protectant through activation of Akt1 and mitochondrial modulation of cysteine proteases. *Circulation* 2002;106:2973–9. [PubMed: 12460881]
- Chong ZZ, Kang JQ, Maiese K. Essential cellular regulatory elements of oxidative stress in early and late phases of apoptosis in the central nervous system. *Antioxid Redox Signal* 2004b;6:277–87. [PubMed: 15025929]
- Chong ZZ, Li F, Maiese K. Attempted cell cycle induction in post-mitotic neurons occurs in early and late apoptotic programs through Rb, E2F1, and caspase 3. *Curr Neurovasc Res* 2006a;3:25–39. [PubMed: 16472123]
- Chong ZZ, Li F, Maiese K. Cellular demise and inflammatory microglial activation during beta-amyloid toxicity are governed by Wnt1 and canonical signaling pathways. *Cell Signal* 2007a;19:1150–62. [PubMed: 17289346]
- Chong ZZ, Li F, Maiese K. Erythropoietin requires NF-kappaB and its nuclear translocation to prevent early and late apoptotic neuronal injury during beta-amyloid toxicity. *Curr Neurovasc Res* 2005b; 2:387–99. [PubMed: 16375720]
- Chong ZZ, Li F, Maiese K. Group I metabotropic receptor neuroprotection requires Akt and its substrates that govern FOXO3a, Bim, and beta-catenin during oxidative stress. *Curr Neurovasc Res* 2006b; 3:107–17. [PubMed: 16719794]
- Chong ZZ, Li F, Maiese K. Oxidative stress in the brain: Novel cellular targets that govern survival during neurodegenerative disease. *Prog Neurobiol* 2005c;75:207–46. [PubMed: 15882775]
- Chong ZZ, Li F, Maiese K. Stress in the brain: novel cellular mechanisms of injury linked to Alzheimer's disease. *Brain Res Brain Res Rev* 2005d;49:1–21. [PubMed: 15960984]
- Chong ZZ, Li F, Maiese K. The pro-survival pathways of mTOR and protein kinase B target glycogen synthase kinase-3beta and nuclear factor-kappaB to foster endogenous microglial cell protection. *Int J Mol Med* 2007b;19:263–72. [PubMed: 17203200]
- Chong ZZ, Lin SH, Maiese K. The NAD⁺ precursor nicotinamide governs neuronal survival during oxidative stress through protein kinase B coupled to FOXO3a and mitochondrial membrane potential. *J Cereb Blood Flow Metab* 2004c;24:728–43. [PubMed: 15241181]
- Chong ZZ, Maiese K. Erythropoietin involves the phosphatidylinositol 3-kinase pathway, 14-3-3 protein and FOXO3a nuclear trafficking to preserve endothelial cell integrity. *Br J Pharmacol* 2007a; 150:839–50. [PubMed: 17339844]
- Chong ZZ, Maiese K. The Src homology 2 domain tyrosine phosphatases SHP-1 and SHP-2: diversified control of cell growth, inflammation, and injury. *Histol Histopathol* 2007b;22:1251–67. [PubMed: 17647198]
- Chugh P, Fan S, Planelles V, Maggirwar SB, Dewhurst S, Kim B. Infection of human immunodeficiency virus and intracellular viral Tat protein exert a pro-survival effect in a human microglial cell line. *J Mol Biol* 2007;366:67–81. [PubMed: 17157319]
- Damjanac M, Rioux BA, Paccalin M, Pontcharraud R, Fauconneau B, Hugon J, Page G. Dissociation of Akt/PKB and ribosomal S6 kinase signaling markers in a transgenic mouse model of Alzheimer's disease. *Neurobiol Dis* 2008;29:354–67. [PubMed: 18023354]
- De Felice FG, Velasco PT, Lambert MP, Viola K, Fernandez SJ, Ferreira ST, Klein WL. Abeta oligomers induce neuronal oxidative stress through an *N*-methyl-D-aspartate receptor-dependent mechanism

- that is blocked by the Alzheimer drug memantine. *J Biol Chem* 2007;282:11590–601. [PubMed: 17308309]
- Dringen R. Oxidative and antioxidative potential of brain microglial cells. *Antioxid Redox Signal* 2005;7:1223–33. [PubMed: 16115027]
- Gomez-Gutierrez JG, Souza V, Hao HY, Montes de Oca-Luna R, Dong YB, Zhou HS, McMasters KM. Adenovirus-mediated gene transfer of FKHRL1 triple mutant efficiently induces apoptosis in melanoma cells. *Cancer Biol Ther* 2006;5:875–83. [PubMed: 16861905]
- Jonsson H, Allen P, Peng SL. Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. *Nat Med* 2005;11:666–71. [PubMed: 15895074]
- Kang JQ, Chong ZZ, Maiese K. Critical role for Akt1 in the modulation of apoptotic phosphatidylserine exposure and microglial activation. *Mol Pharmacol* 2003;64:557–69. [PubMed: 12920191]
- Kawamoto EM, Lepsch LB, Boaventura MF, Munhoz CD, Lima LS, Yshii LM, Avellar MC, Curi R, Mattson MP, Scavone C. Amyloid beta-peptide activates nuclear factor-kappaB through an N-methyl-D-aspartate signaling pathway in cultured cerebellar cells. *J Neurosci Res* 2008;86:845–60. [PubMed: 17969100]
- Li F, Chong ZZ, Maiese K. Microglial integrity is maintained by erythropoietin through integration of Akt and its substrates of glycogen synthase kinase-3beta, beta-catenin, and nuclear factor-kappaB. *Curr Neurovasc Res* 2006;3:187–201. [PubMed: 16918383]
- Li F, Chong ZZ, Maiese K. Vital elements of the wnt-frizzled signaling pathway in the nervous system. *Curr Neurovasc Res* 2005;2:331–40. [PubMed: 16181124]
- Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 2004;21:203–13. [PubMed: 15308101]
- Lin SH, Chong ZZ, Maiese K. Cell cycle induction in post-mitotic neurons proceeds in concert with the initial phase of programmed cell death in rat. *Neurosci Lett* 2001;310:173–7. [PubMed: 11585595]
- Ludikhuize J, de Launay D, Groot D, Smeets TJ, Vinkenoog M, Sanders ME, Tas SW, Tak PP, Reedquist KA. Inhibition of forkhead box class O family member transcription factors in rheumatoid synovial tissue. *Arthritis Rheum* 2007;56:2180–91. [PubMed: 17599731]
- Maiese K. Diabetic stress: new triumphs and challenges to maintain vascular longevity. *Expert Rev Cardiovasc Ther* 2008a;6:281–4. [PubMed: 18327989]
- Maiese K. Triple play: promoting neurovascular longevity with nicotinamide, WNT, and erythropoietin in diabetes mellitus. *Biomed Pharmacother* 2008b;62:218–232. [PubMed: 18342481]
- Maiese K, Chong Z, Li F. Reducing oxidative stress and enhancing neurovascular longevity during diabetes mellitus. In: Maiese K, editor. *Neurovascular Medicine: Pursuing Cellular Longevity for Healthy Aging*. Oxford University Press; New York, NY: 2009a.
- Maiese K, Chong ZZ. Insights into oxidative stress and potential novel therapeutic targets for Alzheimer disease. *Restor Neurol Neurosci* 2004;22:87–104. [PubMed: 15272144]
- Maiese K, Chong ZZ. Nicotinamide: necessary nutrient emerges as a novel cytoprotectant for the brain. *Trends Pharmacol Sci* 2003;24:228–32. [PubMed: 12767721]
- Maiese K, Chong ZZ, Hou J, Shang YC. Erythropoietin and oxidative stress. *Curr Neurovasc Res* 2008a; 5:125–142. [PubMed: 18473829]
- Maiese K, Chong ZZ, Li F, Shang YC. Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies. *Prog Neurobiol* 2008b;85:194–213. [PubMed: 18396368]
- Maiese K, Chong ZZ, Shang YC. OutFOXing disease and disability: the therapeutic potential of targeting FoxO proteins. *Trends Mol Med* 2008c;14:219–227. [PubMed: 18403263]
- Maiese K, Chong ZZ, Shang YC. “Sly as a FOXO”: new paths with forkhead signaling in the brain. *Curr Neurovasc Res* 2007;4:295–302. [PubMed: 18045156]
- Maiese K, Chong ZZ, Shang YC, Hou J. Clever cancer strategies with FoxO transcription factors. *Cell Cycle* 2008d;7:3829–39. [PubMed: 19066462]
- Maiese K, Chong ZZ, Shang YC, Hou J. FoxO proteins: cunning concepts and considerations for the cardiovascular system. *Clin Sci (Lond)* 2009b;116:191–203. [PubMed: 19118491]
- Maiese K, Chong ZZ, Shang YC, Hou J. Rogue proliferation versus restorative protection: where do we draw the line for Wnt and forkhead signaling? *Expert Opin Ther Targets* 2008e;12:905–16. [PubMed: 18554157]

- Maiese K, Li F, Chong ZZ. New avenues of exploration for erythropoietin. *JAMA* 2005;293:90–5. [PubMed: 15632341]
- Majd S, Zarifkar A, Rastegar K, Takhshid MA. Different fibrillar Abeta 1–42 concentrations induce adult hippocampal neurons to reenter various phases of the cell cycle. *Brain Res* 2008;1218:224–9. [PubMed: 18533137]
- Mallat M, Marin-Teva JL, Cheret C. Phagocytosis in the developing CNS: more than clearing the corpses. *Curr Opin Neurobiol* 2005;15:101–7. [PubMed: 15721751]
- Martinez-Contreras A, Huerta M, Lopez-Perez S, Garcia-Estrada J, Luquin S, Beas Zarate C. Astrocytic and microglia cells reactivity induced by neonatal administration of glutamate in cerebral cortex of the adult rats. *J Neurosci Res* 2002;67:200–10. [PubMed: 11782964]
- Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000;403:98–103. [PubMed: 10638761]
- Newman M, Musgrave IF, Lardelli M. Alzheimer disease: amyloidogenesis, the presenilins and animal models. *Biochim Biophys Acta* 2007;1772:285–97. [PubMed: 17208417]
- Obexer P, Geiger K, Ambros PF, Meister B, Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. *Cell Death Differ* 2007;14:534–47. [PubMed: 16888645]
- Orlacchio A, Bernardi G, Martino S. RNA interference as a tool for Alzheimer's disease therapy. *Mini Rev Med Chem* 2007;7:1166–76. [PubMed: 18045220]
- Power JH, Asad S, Chataway TK, Chugini F, Manavis J, Temlett JA, Jensen PH, Blumbergs PC, Gai WP. Peroxiredoxin 6 in human brain: molecular forms, cellular distribution and association with Alzheimer's disease pathology. *Acta Neuropathol* 2008;115:611–22. [PubMed: 18386021]
- Sela U, Dayan M, HersHKoviz R, Cahalon L, Lider O, Mozes E. The negative regulators Foxj1 and Foxo3a are up-regulated by a peptide that inhibits systemic lupus erythematosus-associated T cell responses. *Eur J Immunol* 2006;36:2971–80. [PubMed: 17051618]
- Sheng JG, Mrak RE, Griffin WS. Neuritic plaque evolution in Alzheimer's disease is accompanied by transition of activated microglia from primed to enlarged to phagocytic forms. *Acta Neuropathol (Berl)* 1997;94:1–5. [PubMed: 9224523]
- Simak J, Holada K, Vostal JG. Release of annexin V-binding membrane microparticles from cultured human umbilical vein endothelial cells after treatment with camptothecin. *BMC Cell Biol* 2002;3:11. [PubMed: 12052248]
- Soares MM, King SW, Thorpe PE. Targeting inside-out phosphatidylserine as a therapeutic strategy for viral diseases. *Nat Med* 2008;14:1357–62. [PubMed: 19029986]
- Sun ZK, Yang HQ, Pan J, Zhen H, Wang ZQ, Chen SD, Ding JQ. Protective effects of erythropoietin on tau phosphorylation induced by beta-amyloid. *J Neurosci Res* 2008;86:3018–27. [PubMed: 18512763]
- Troy CM, Rabacchi SA, Xu Z, Maroney AC, Connors TJ, Shelanski ML, Greene LA. Beta-amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. *J Neurochem* 2001;77:157–64. [PubMed: 11279271]
- Vaisid T, Barnoy S, Kosower NS. Calpastatin overexpression attenuates amyloid-beta-peptide toxicity in differentiated PC12 cells. *Neuroscience* 2008;156:921–31. [PubMed: 18786620]
- Williams K, Schwartz A, Corey S, Orandle M, Kennedy W, Thompson B, Alvarez X, Brown C, Gartner S, Lackner A. Proliferating cellular nuclear antigen expression as a marker of perivascular macrophages in simian immunodeficiency virus encephalitis. *Am J Pathol* 2002;161:575–85. [PubMed: 12163382]
- Wu S, Xing W, Peng J, Yuan X, Zhao X, Lei P, Li W, Wang M, Zhu H, Huang B, Huang L, Shen G. Tumor transfected with CCL21 enhanced reactivity and apoptosis resistance of human monocyte-derived dendritic cells. *Immunobiology* 2008;213:417–26. [PubMed: 18472050]

Fig. (1). Amyloid (A β_{1-42}) leads to cell injury, DNA degradation, and membrane phosphatidylserine (PS) exposure in microglial cells

(A) Microglial cells were exposed to A β_{1-42} (10 μ M) and cell survival was determined 24 hours after A β_{1-42} by using the trypan blue exclusion method. Top panel: Representative images illustrate that A β_{1-42} leads to significant trypan blue uptake. Bottom panel: Microglial survival was progressively decreased with increased concentrations of A β_{1-42} (* P <0.01 vs. untreated control). Each data point represents the mean and SEM from 6 experiments. (B) Top panel: Representative images illustrate that A β_{1-42} (10 μ M) leads to apoptotic DNA fragmentation (dark nuclei) with TUNEL stain and membrane PS externalization (green fluorescence) with annexin V twenty-four hours following A β_{1-42} exposure. Bottom panel: A β_{1-42} (10 μ M) application (10 μ M) significantly increased percent DNA fragmentation and PS exposure in microglia (* P <0.01 vs. untreated control). Each data point represents the mean and SEM from 6 experiments.

Fig. (2). Early microglial activation and proliferation occurs following amyloid ($A\beta_{1-42}$) exposure Representative images and quantitative analysis illustrate that PCNA and BrdU expression is significantly and rapidly increased in microglia at 3 hours and 6 hours following $A\beta_{1-42}$ (10 μ M) exposure (* $P < 0.01$ vs. control). In all cases, control = untreated cells. Each data point represents the mean and SEM from 6 experiments.

Fig. (3). Phosphorylation FoxO3a is lost allowing its translocation to the cell nucleus during amyloid (Aβ₁₋₄₂) exposure

In **A** and **B**, microglial protein extracts (50 μg/lane) were immunoblotted with anti-phosphorylated-FoxO3a (p-FoxO3a, Ser²⁵³) or anti-total FoxO3a at 6 hours following Aβ₁₋₄₂ (10 μM) administration. Phosphorylated (active) FoxO3a (p-FoxO3a) expression is significantly decreased 6 hours following application of Aβ₁₋₄₂ (10 μM) but total FoxO3a remains unchanged (**P*<0.01 vs. control). In **C** and **D**, microglia were followed at 6 hours after Aβ₁₋₄₂ (10 μM) administration with immunofluorescent staining for FoxO3a (Texas-red). Nuclei of microglia were counterstained with DAPI. In merged images, control cells have readily visible nuclei (white in color) that illustrate absence of FoxO3a in the nucleus. In contrast, merged images after Aβ₁₋₄₂ (10 μM) exposure are not visible (red in color) and demonstrate translocation of FoxO3a to the nucleus. Quantification of the intensity of FoxO3a nuclear staining was performed using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image>) (**P*<0.01 vs. control). Control = untreated microglia.

Fig. (4). Removal of FoxO3a through gene silencing protects microglia during amyloid ($A\beta_{1-42}$) exposure

In **A** and **B**, microglial protein extracts (50 μ /lane) were immunoblotted with anti-phosphorylated-FoxO3a (p-FoxO3a, Ser²⁵³) or anti-total FoxO3a at 6 hours following $A\beta_{1-42}$ (10 μ M) administration. Transfection with FoxO3a siRNA (siRNA) significantly reduced expression of total FoxO3a alone or following $A\beta_{1-42}$ (10 μ M) administration (* P <0.01 vs. control). In **C** and **D**, gene silencing with FoxO3a siRNA (siRNA) significantly blocked microglial membrane injury assessed by trypan blue staining 24 hours after $A\beta_{1-42}$ (10 μ M) administration. FoxO3a siRNA alone was not toxic and scrambled siRNA did not

protect cells during A β_{1-42} (10 μ M) exposure (* P <0.01 vs. untreated cells). Each data point represents the mean and SEM from 6 experiments.

Fig. (5). Gene silencing of FoxO3a blocks apoptotic DNA fragmentation and phosphatidylserine (PS) externalization

In **A** and **B**, representative images and quantitative analysis illustrate gene silencing with FoxO3a siRNA (siRNA) significantly blocked microglial genomic DNA degradation assessed by TUNEL and membrane PS externalization 24 hours after $A\beta_{1-42}$ (10 μ M) administration. FoxO3a siRNA alone was not toxic and scrambled siRNA did not protect cells during $A\beta_{1-42}$ (10 μ M) exposure (* P <0.01 vs. untreated cells). Each data point represents the mean and SEM from 6 experiments.

Fig. (6). Transfection with FoxO3a siRNA prevents early microglial activation and proliferation occurs following amyloid (A β ₁₋₄₂) exposure

In **A** and **B**, representative images and quantitative analysis demonstrate that gene silencing with FoxO3a siRNA (siRNA) decreases the expression of PCNA and the uptake of BrdU at 6 hours following A β ₁₋₄₂ (10 μ M) exposure (* P <0.01 vs. control or untreated cells). In all cases, control = untreated cells. Each data point represents the mean and SEM from 6 experiments.

Fig. (7). Caspase 3 activity in microglial cells is controlled by FoxO3a during amyloid ($A\beta_{1-42}$) exposure

In **A** and **B**, microglial protein extracts (50 μ g/lane) were immunoblotted with anti-cleaved caspase 3 product (active caspase 3) at 6 hours following $A\beta_{1-42}$ (10 μ M) administration. $A\beta_{1-42}$ (10 μ M) administration markedly increased cleaved caspase 3 expression, but transfection with FoxO3a siRNA (siRNA) significantly reduced the activity of caspase 3 following $A\beta_{1-42}$ (10 μ M) administration. Gene silencing with FoxO3a siRNA (siRNA) significantly prevented cleaved caspase 3 expression 6 hours after $A\beta_{1-42}$ (10 μ M) administration. FoxO3a siRNA alone was not toxic and scrambled siRNA did not reduce cleaved caspase 3 expression during $A\beta_{1-42}$ (10 μ M) exposure (* P <0.01 vs. control). Each data point represents the mean and SEM from 6 experiments.