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Age-related differences in NFκB translocation and Bcl-2/Bax ratio caused by TNFα and Abeta42 promote survival in middle-age neurons and death in old neurons

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Abstract

Alzheimer's disease is associated with an age-related accumulation of Abeta and inflammation. The inflammatory mediator, TNFα activates a signaling cascade involving NFκB translocation to the nucleus and a beneficial or detrimental transcriptional response, depending on the age of the neurons and the type of stress applied. Relative to treatment with Abeta42 alone, previously we found that TNFα plus Abeta42, applied to old rat neurons (24 month) is toxic, while the same treatment of middle-age neurons (10 month) is protective. In contrast to improved survival of middle-age rat cortical neurons, neurons from old rats are killed by TNFα plus Abeta42 despite greater p50 nuclear translocation. In middle-age neurons, blocking TNFR1 does not affect NFκB translocation, whereas blocking TNFR2 results in an increase in NFκB translocation. For old neurons, blocking either receptor, does not change NFκB translocation, but improves cell survival. To account for these effects on cell viability in response to TNF + Abeta, measures of the Bcl-2/Bax ratio positively correlate with survival. In the setting of old neurons, these results suggest that overactivated nuclear translocation of NFκB and lower Bcl-2 levels promote death that is reduced by inhibition of either TNFR1 or R2.

Keywords

TNFR1; TNFR2; blocking antibodies; cortical neurons

Introduction

The inflammatory response in the brain of Alzheimer's disease (AD) patients may exacerbate neurodegeneration (Akiyama et al., 2000). Increases in inflammatory cytokines in the brain with age (Davies et al., 2003; Knoblach et al., 1999; Gemma et al., 2002), likely contribute to neurodegeneration. Elevated beta-amyloid (Abeta) may be another factor contributing to inflammation. Abeta forms soluble oligomers and insoluble fibrillar aggregates that deposit in the brain forming senile plaques that are associated with neurodegeneration. Abeta both

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directly and indirectly activates microglia. The deposition of Abeta activates reactive microglia and other inflammatory responses (Sheng et al., 1997). Using the scavenger receptor, Abeta fibrils attach to microglia, which in turn, release TNF α and produce reactive oxygen species (ROS) (El Khoury et al., 1998; Johnstone et al., 1999). Microglia are chronically activated due to the presence of Abeta, therefore chronically releasing TNF α . TNF α is neurotoxic and with other factors elicits neuronal cell death (Combs et al., 2001; Kang et al., 2001). In comparing, cerebrospinal fluid (CSF) levels of TNF α , TNF β , IL-1 β , IL-1 receptor antagonist (IL1ra), and IL-6 in AD patients and healthy age-matched controls, TNF α was the only cytokine found to be elevated in AD patients (Tarkowski et al., 2003). There was no significant difference between CSF levels of the two groups for TNF β , IL-1 β , IL-6; however, IL-1ra levels were lower in AD patients. We have previously shown age-related increases in TNF α production by rat microglia in response to Abeta (Viel et al., 2001) and age-related toxicity from the combination of TNF α plus Abeta40 or Abeta42 (Patel and Brewer, submitted).

Recently, Abeta has been shown to activate NF κ B through the tumor necrosis factor receptor 1 (TNFR1) signaling cascade that results in apoptosis of neurons (Li et al., 2004; Valerio et al., 2006). However, others have shown that NF κ B activates anti-apoptotic genes and protects neurons from excitotoxicity and ischemic injury in the mature brain (Schneider et al., 1999; Pennypacker et al., 2001; Bhakar et al., 2002; Mattson, 2005). We hypothesize that age-related down-regulation to the TNFR1 and TNFR2 signaling results in failure of NF κ B translocation to the nucleus that fails to provide a neuroprotective response against Abeta42 toxicity by TNF α . Our results show that both middle-age and old neurons translocate NF κ B to the nucleus in response to TNF α plus Abeta42 treatment, but NF κ B accumulates in old neuron nuclei. This effect is unchanged when TNFR1 is blocked in both middle-age and old neurons. When TNFR2 is blocked, middle-age neurons increase translocation of NF κ B, but old neurons do not. These results suggest that promotion of survival or death in the presence of TNF + Abeta depends on the amount of nuclear NF κ B and Bcl-2/Bax ratio.

Materials and Methods

Adult Neuronal Cell Culture

Cortical neurons were isolated from 10-12 month old (middle-age) and 24-25 month old (old) male rats (Fisher 344, Harlan, Indianapolis, IN; Brewer, 1997; Brewer and Torricelli, 2007). Isoflurane (Baxter, Deerfield, IL) was used to anaesthetize the animals. Rats were decapitated and their brains removed. The cortices were isolated in Hibernate A (www.BrainBitsLLC.com)/ 2% B27/0.5 mM Glutamax (Invitrogen, Carlsbad, CA) and chopped into 500 μ m slices (McIlwain tissue chopper, Mickle Laboratory Engineering, Guilford, UK). Next, the tissue was placed in 5 ml Neurobasal A/ 2% B27/0.5 mM Glutamax for 8 minutes in a shaking water bath at 30°C. The tissue was then treated with 2 mg/ml papain (Worthington, Lakewood, NJ) for 30 minutes in a shaking water bath at 30°C. The cortical tissue was triturated 3 times. Using two 4 ml density gradients of Optiprep (Sigma, St. Louis, MO), neurons were separated from other non-neuronal cells by centrifugation at 800 $\times$ g for 15 minutes. Fractions containing neurons were collected and mixed with 5 ml of Hibernate A/ 2% B27/0.5 mM Glutamax and centrifuged at 200 $\times$ g for 2 minutes. The supernatant was aspirated and the pellet was resuspended in 5 ml of Hibernate A/2% B27/0.5 mM Glutamax and centrifuged a third time at 200 $\times$ g for 2 minutes. The pellet was resuspended in 8 ml of Neurobasal A/2% B27/0.5 mM Glutamax and viable cells counted by trypan blue exclusion. Cells were plated in Neurobasal A medium supplemented with 2% B27/0.5 mM Glutamax, 1 μ g/ml Gentamycin (Invitrogen) and 5 ng/ml human fibroblast growth factor 2 (FGF2, Invitrogen). Neurons were plated on glass coverslips (Carolina Biologicals, Burlington, NC), coated with 100 μ g/ml poly-d-lysine (Sigma), at a density of 32,000 cells/cm². The coverslips were either 1 cm² for imaging or 12 cm² for gel shift assay. Adult cultures were incubated at

37°C in 5% CO₂ and 9% O₂ (Thermo-Forma, Waltham, MA) until 8-10 days in vitro. Cultures were immunostained with neuronal and glial markers to determine percentage of neurons (Brewer, 1997). Adult cultures were > 80% neurons (data not shown).

Neuron treatments

Cultures were treated with TNF α (0.01- 1 μ g/ml Peprotech, Rocky Hill, NJ) and/or 10 μ M fibrillar Abeta (1-42; US Peptides, Rancho Cucamonga, CA). Fibrillar Abeta42 was prepared by dissolving solid peptide in water at 2.5 mM and diluting in Dulbecco's phosphate-buffered saline (dPBS, Invitrogen) to a final concentration of 0.5 mM. Next the Abeta42 was incubated for 3 days at 37°C for aggregation before use (Simmons et al., 1994). Adult cortical cultures at 8-10 days in vitro were placed in fresh medium containing Neurobasal A/B27/Glutamax and TNF α . Untreated controls also underwent 100 % medium changes. The cells were incubated for 24 hours at 37°C in 5% CO₂ and 9% O₂. After 24 hours, 10 μ M Abeta was added to the cultures and were incubated for another 24 hours at 37°C in 5% CO₂ and 9% O₂. The cultures were now used in viability assays, fixed for immunocytochemistry, and nuclear protein extraction. Middle-age and old neurons were treated with TNF α for 24 hrs followed by 24 hrs of Abeta42 treatment, as before (Patel and Brewer, in press). Experiments using blocking antibodies against TNFR1 (Anti-mouse TNFR1, R&D Systems, Minneapolis, MN) and TNFR2 (Anti-mouse TNFR2, Biolegend, San Diego, CA), cultures were treated with the antibody for 1 hour before TNF α was added. Antibody concentrations were chosen by determining the dose that produced the highest survival of old neurons.

Immunocytochemistry of NF κ B, Bcl-2 and Bax

Neurons were treated as described above and then fixed in 4% paraformaldehyde, blocked with 5% normal goat serum (NGS, Invitrogen,) and 0.5% TX-100 (Sigma) in phosphate buffered saline (PBS). Antibodies were diluted in 5% NGS and 0.05% TX-100 in PBS. Primary rabbit antibody against the p50 subunit of NF κ B (1:500 dilution; QED Bioscience, San Diego, CA) or mouse anti-Bcl-2 IgG (1:150 dilution, BD Transduction, San Diego, CA) and rabbit anti-mouse Bax antiserum (1:1000 dilution, BD Pharmingen, San Diego, CA) were added to the neurons for overnight incubation at 4°C. Twenty-four hours later, after four rinses with PBS, the secondary antibody, Alexa fluor 568 goat anti-rabbit (1:2000 dilution; Molecular Probes, Eugene, OR) and/or Alexa fluor 488 anti-mouse IgG (1:1000, Molecular Probes) were added for 60 minutes at room temperature. Duplicate coverslips were rinsed two times with PBS and incubated in 1 μ g/ml Bisbenzamide (Sigma) for 2 minutes, followed by two rinses with PBS. Coverslips were mounted onto a clean glass microscope slide with Aquamount (Fisher). These mounted slides were imaged using an Olympus epifluorescence microscope under 60 $\times$ oil objective (N.A. 1.42) with Dapi and Tritc filters and digital Q-imaging camera (Media Cybernetics, Silver Springs, MD). Exposure time and gain were determined by finding the brightest neurons within treatment and age. Two sets of six consecutive fields per coverslips were imaged at the set exposure time and gain. The images were analyzed using Image-Pro Plus Software (Media Cybernetics). Density and area threshold ranges were chosen based on the lowest density and area values within treatment and age and kept constant for all neurons imaged. Each individual cell was analyzed for density and area. NF κ B translocation to the nucleus was analyzed by first using the bisbenzamide images to determine the outline of the nucleus, and then the outlines were overlaid onto the p50 images. Density and area were determined only within the nuclear outlines. Mean background cellular fluorescence of non-specific binding of secondary antibodies alone was subtracted from all images to account for autofluorescence of neurons. Fluorescence from lipofuscin was either far below that of specific antibody labels and/or was lost by turn-over during regeneration. The amount of cytoplasm imaged was minimal because of the high numerical aperture objective used to selectively focus on the nucleus.

Nuclear extraction preparation

Middle-age and old cortical cultures at 9 days in vitro were treated with 100 ng/ml TNF only, 10 μ M Abeta42 only or 100 ng/ml TNF plus 10 μ M Abeta42 together. Nuclear extraction was conducted based on Dignam et al. (1983). Cultures were rinsed twice in ice-cold PBS. Cytoplasmic extracts were prepared in buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 % nonidet P 40 (NP-40), and protease inhibitor cocktail (Sigma)). The cells were scraped off coverslips in Buffer A, vortexed for 15 seconds, and incubated on ice for 10 minutes. Next 10% NP-40 is added to each tube (final NP-40 concentration = 1%), vortexed for 5 seconds, incubated on ice for 1 minutes and centrifuged at 16,000 $\times$ g for 5 minutes at 4°C. The supernatant was the cytoplasmic fraction and the pellet was the nuclear fraction. The pellet was resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 15 mM MgCl₂, 0.5 mM DTT, 0.2 mM EDTA and protease inhibitor cocktail), vortexed for 30 seconds at highest setting and rotated at 4°C for 30 minutes. Next the nuclei were centrifuged at 16,000 $\times$ g for 15 minutes at 4°C. The supernatant contains nuclear proteins and is used for electrophoretic mobility shift assays. Protein was quantified using the Micro BCA protein assay kit (Pierce).

Annealing oligonucleotides for electrophoretic mobility shift assay

Sense and anti-sense oligos were purchased from Bioneer (Alameda, CA). The sense strand was biotinylated. The following were NF κ B sense strand sequence: 5' ACTTGAGGGGACTTTCCAGGC 3' and antisense sequence 5' GCCTGGGAAAGTCCCCTCAAGT 3'. The oligonucleotides were reconstituted in 10 mM Tris, 1 mM EDTA, and 50 mM NaCl pH 8.0. The complementary strand oligonucleotides were mixed together at 1:1 molar concentrations and annealed in a thermocycler. Each set of oligos were incubated at 95°C for 5 minutes, followed by 70 cycles at which the temperature decreased by 1°C every cycle. At the end of the cycles the oligos were aliquoted and stored at -70°C.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were used to determine NF κ B translocation to the nucleus. Ten micrograms of protein were incubated with 280 fmol of either double stranded biotinylated oligos for NF κ B, 10 μ g/ml BSA, 2.5% glycerol, 1 μ g Poly dI.dC, 0.1% Triton-x for 1 hour at room temperature. All solutions were from Pierce's Lightshift Chemiluminescent EMSA kit (Rockford, IL) and samples were electrophoresed according to manufacturer's instructions. Samples were electrophoresed on 6% nondenaturing gel at 200 volts for 1 hour and transferred to charged nylon membrane for 1.5 hours at 200 mA. The membrane was UV cross-linked for 15 minutes and blocked for 15 minutes, followed by incubation with streptavidin-horse radish peroxidase conjugate in blocking buffer. The membrane was then washed for 20 minutes and then incubated for 5 minutes in substrate equilibration buffer. Next it was incubated for 5 minutes in luminal/enhancer solution and peroxide solution and visualized using a charged-coupled device camera (Hitachi Genetic Systems, MiraiBio Inc., Alameda, CA). Bands were quantified using Image-Pro Plus software. Super shifts were performed to verify p50 subunit using antibody against p50 (data not shown, Santa Cruz Biotechnology, Santa Cruz, CA).

Summary and Statistical Methods

Experiments were conducted on cultured cortical neurons and cortical extracts from 10 month, and 25 month old rats. Each culture consisted of 16,000 to 1.3 million neurons depending on surface area of plating surface. Student's t-test was conducted to determine effect of treatment within each age. Mean and Standard errors are reported.

Results

Our aging neuron model successfully extracts live cortical neurons from middle-age (11 month) or old (24 month) Fisher rats (Brewer, 1997; 1998; Brewer & Torricelli, 2007), near their median lifespan (Solleveld et al., 1984). Similar percentages of neurons survive the dissociation process to regenerate axons and dendrites (Brewer, 1997; 1998; Patel and Brewer 2003). Compared to middle-age neurons, the neurons from old animals take up glucose at the same rate (Patel and Brewer, 2003), have similar passive membrane properties and generation of action potentials (Evans et al., 1998). The resting membrane potentials of old and middle-age neurons are similar (Cady et al., 2001). Therefore, we are not isolating old neurons that are noticeably more fragile or deficient in their ability to regenerate axons and dendrites. Based on previous observations that 100 ng/ml TNF α protected against killing by 10 μ M Abeta42 in middle-age, but enhanced toxicity in old neurons, we hypothesized that NF κ B translocation from the cytoplasm to the nucleus would be greater for middle-age neurons than old neurons when TNF α , Abeta42, or TNF α plus Abeta42 were added. We examined NF κ B translocation to the nucleus by nuclear immunoreactivity of the active subunit p50 (Fig 1). Both middle-age and old neurons show greater p50 nuclear translocation than untreated controls (Fig 1A-H). Digital analysis of these and other images (Fig 1M) shows significantly greater p50 signal in the nucleus for middle-age neurons compared to old neurons. However, when treated with 100 ng/ml TNF α plus 10 μ M Abeta42 (Fig 1M), both middle-age and old neurons show a significant increase in p50 immunoreactivity in the nucleus. This increase in p50 immunoreactivity is significantly greater for the old neurons than middle-age neurons ($p=0.02$). Compared to untreated neurons, nuclear p50 signal increased 20% in middle-age neurons and 60 % in old neurons treated with TNF α plus Abeta42. The results also show that old neurons are capable of NF κ B activation by p50 translocation to the nucleus.

We also examined NF κ B translocation by binding to the DNA consensus sequence in nuclear extracts. We expected to find results similar to the immunocytochemistry experiment. However, the crude nature of nuclear extracts showed that middle-age neurons have greater binding activity than old neurons when untreated, treated with TNF α or TNF α plus Abeta42 (Fig 2). When middle-age and old neurons were treated with Abeta42 alone they show similar levels of NF κ B translocation. We used blocking antibodies to determine if blocking TNF α binding to TNFR1 or TNFR2 would interfere with neuron survival. We hypothesized that blocking TNFR1 would result in greater cell death for TNF α plus Abeta42 treated old neurons, whereas blocking TNFR2 would result in greater cell survival. In Fig. 3A, middle-age neurons show a significant reduction in cell death when treated with blocking antibodies for TNFR1 ($p=0.03$) or TNFR2 ($p=0.05$). Old neurons also show significant reductions in cell death when treated with either TNFR1 ($p<0.01$) or TNFR2 ($p<0.01$) blocking antibodies. This suggests that both middle-age and old neurons need both receptors for the activation of cell death signals. When either receptor was blocked, cell death signals were decreased.

We examined NF κ B translocation to the nucleus, as in Figs. 1, but in the presence of blocking antibodies. The activation of TNFR has been shown to result in NF κ B translocation to the nucleus (Miyamoto et al., 1994; Hsu et al., 1995). We hypothesized that blocking TNFR1 for either age would result in lower nuclear p50 immunoreactivity and blocking TNFR2 would not result in a reduction in p50 immunoreactivity for either age compared to unblocked controls. Fig 3B shows that when TNFR1 blocking antibody acted on middle-age and old neurons, p50 nuclear immunoreactivity did not change for either age. When TNFR2 blocking antibody is used, middle-age neurons increased p50 nuclear immunoreactivity, but old neurons did not show any change in p50 nuclear immunoreactivity.

To explain the greater toxicity of old neurons treated with TNF + Abeta, we reasoned that neuroprotective Bcl-2 would be downregulated in old neurons compared to middle-age neurons

and/or proapoptotic Bax immunoreactivity would be greater in old neurons than middle-age neurons. We examined Bcl-2 and Bax immunoreactivity following similar treatments (Fig. 4). In Fig. 4A-B, untreated old neurons show higher Bcl-2 immunoreactivity than untreated middle-age neurons. After treatment with TNF + Abeta, middle-age neurons have similar levels of Bcl-2 as the untreated controls (Fig. 4A, C), however, the old neurons after TNF + Abeta treatment downregulated Bcl-2 levels (Fig 4B, D). Digital analysis of these and other images show age-related and treatment differences in Bcl-2 and Bax (Fig. 4E-H). For middle-age neurons treated with TNF or Abeta, Bcl-2 or Bax levels did not change compared to untreated controls (Fig. 4E). Compared to middle-age neurons treated with TNF, middle-age neurons treated with TNF + Abeta ($p=0.05$) had lower levels of Bax, while Bcl-2 levels did not change. This lowering of proapoptotic Bax in middle-age neurons treated with TNF + Abeta correlates with the observed neuroprotection for these conditions (Fig. 3A). Compared to middle-age neurons treated with Abeta, the combined treatment of TNF + Abeta elicited lower levels of Bcl-2 ($p=0.05$). For middle-age neurons, treatment with Abeta alone produced higher Bcl-2 levels and lower Bax levels that were now significantly different ($p=0.001$). Compared to TNF + Abeta treatment alone, treatment of middle-age neurons with R1 blocking antibody and TNF + Abeta, elevates Bcl-2 ($p=0.01$) and Bax levels ($p<0.001$, Fig. 4E).

In contrast to middle-age neurons, untreated old neurons showed significantly less Bax than Bcl-2 ($p<0.001$, Fig. 4F). Compared to untreated old neurons, Bcl-2 immunoreactivity is significantly downregulated following treatment with TNF alone ($p=0.01$), Abeta alone ($p=0.002$), TNF + Abeta alone ($p<0.0001$), TNFR1 blocking antibody with TNF + Abeta ($p=0.03$) or TNFR2 blocking antibody with TNF + Abeta ($p=0.02$). Compared to Abeta treatment of old neurons, TNF + Abeta treatment of old neurons significantly lowered Bcl-2 levels ($p=0.01$). Adding TNFR1 blocking antibody with TNF + Abeta significantly raised Bcl-2 levels compared to only TNF + Abeta treatment, suggesting a role for TNFR1 in lowering Bcl-2 ($p<0.001$). Bcl-2 levels were significantly higher than Bax levels when old neurons were treated with TNF ($p=0.02$) or Abeta ($p=0.002$, Fig. 4F). However, Bax levels were not significantly affected by any treatment of old neurons.

The same data was rearranged for easier age-related comparisons of Bcl-2 (Fig. 4G) and Bax (Fig. 4H). Compared to untreated middle-age neurons, untreated old neurons had significantly higher Bcl-2 immunoreactivity ($p=0.01$, Fig. 4G). All other treatments reversed this age-related ratio for Bcl-2 and Bax. Compared to untreated middle-age neurons, Bax levels were not different for untreated old neurons. Bax levels in middle-age neurons after TNF treatment were higher than those in old neurons ($p=0.02$) or after TNFR1 blocking with TNF + Abeta treatment ($p=0.04$, Fig. 4H). Bax levels between middle-age and old neurons did not change with other treatments.

Since TNF receptor activation of NF κ B translocation did not easily account for the effects on viability of old neurons, we examined correlations of viability to Bcl-2/Bax ratios. Fig. 4I shows that survival positively correlated with Bcl-2/Bax ratio for nearly all experimental conditions, except three middle-age conditions and TNF treatment of old neurons. Bax levels did not change in middle-age neurons either treated with Abeta or untreated controls. There was an increase in the Bcl-2/Bax ratio with Abeta treatment. Based on previous work (Patel & Brewer, in press), both TNFR1 and TNFR2 were higher in middle-age than in old neurons treated with TNF plus Abeta. This shows that both prosurvival and proapoptotic proteins are critical components of the TNF signaling pathway along with NF κ B translocation.

Discussion

Our data are consistent with the hypothesis that age-related changes to the TNFR1 and TNFR2 signaling results increased NF κ B translocation as p50 translocation to the nucleus that provides

neuroprotection to middle-age neurons, but the same translocation in old neuron fails to protect against TNF α plus Abeta42 toxicity. Untreated old neurons show significantly higher Bcl-2 levels compared to middle-age neurons and other experimental conditions. The immunoreactive Bcl-2/Bax ratio positively correlates with cell survival for middle-age and old neurons treated with Abeta, TNF α + Abeta or R2 blocking antibody with TNF α + Abeta. Others have shown that Abeta toxicity in neuroblastoma cells occurs by Bcl-2 downregulation and upregulated Bax expression (Clementi et al., 2006). Elevated Bcl-2 in old neurons greatly decreased following treatment with TNF α + Abeta and is only partly restored by blocking TNFR1. The positive correlation between survival and Bcl-2/Bax ratio suggests that levels of those proteins exert stronger control over viability in old neurons than does NF κ B translocation to the nucleus. Fig. 5 summarizes the age-related differences between middle-age and old neurons after TNF α + Abeta treatment. Old neurons increase NF κ B translocation to the nucleus, but downregulate Bcl-2/Bax ratio, which correlates with greater old neuron death. As discussed below, previous work has shown old neurons fail to upregulate TNFR1 and TNFR2 receptors (Patel and Brewer, submitted), increase ROS (Parihar and Brewer, 2007) and oxidized redox levels (Parihar et al., in revision) in comparison to middle-age neurons.

Experiments for nuclear translocation of NF κ B were performed by immunostaining neurons for p50 antibody. Middle-age and old neurons treated with either TNF α or Abeta42 alone failed to translocate NF κ B above untreated levels. It is possible that neurons have constitutive NF κ B activation (Meffert et al., 2003); therefore we did not see a difference in p50 translocation when the neurons were treated with proven activators of NF κ B in other cell types. Old and middle-age neurons both show significant up-regulation of p50 translocation when treated with TNF α plus Abeta42. Old neurons show greater NF κ B translocation than middle-age neurons treated with TNF α plus Abeta42. The larger amount of NF κ B translocation in old neurons versus middle-age in response to TNF α + Abeta shows the capacity of NF κ B is patent in old neurons (Fig 1M), but does not account for the loss of viability in old neurons. Fig. 4F and I show that the same TNF α + Abeta treatment that leaves the Bcl-2/Bax ratio unchanged in middle-age neurons greatly reduces the neuroprotective Bcl-2 in old neurons.

The differences between the immunocytochemistry experiments and gel shift may be due to the differences in sensitivity of either technique. The immunocytochemistry is a more sensitive and spatially more specific method than a gel shift assay. The homogenized average of gel shift assays requires a large population of cells and therefore, may not be sensitive to subtle differences in activation between age and treatment groups. Antibody binding for immunocytochemistry is a stable reaction compared to DNA binding activity of gel shift assays. The NF κ B and oligonucleotide complex is sensitive to temperature, pH, protease activity, and salt concentration, therefore may produce variability in signal between experimental conditions.

We conducted viability experiments using TNFR1 and TNFR2 blocking antibodies. Blocking antibodies were added to the adult cultures prior to TNF α and Abeta42 addition. When either TNFR1 or TNFR2 blocking antibodies were added, cell death decreased for both ages. However, old neurons showed a greater decrease in cell death than middle-age neurons, which suggests that old neurons are more dependent on the ratio of surface receptor expression than middle-age neurons. This suggests that both ages need activation of both receptors for apoptosis or that p50 is inactive, possibly because it dimerizes with an inhibitory p65 rather than an activating p50 homodimer or that bcl-2 sites are available for activation in middle-age neurons, but blocked epigenetically in old neurons. The protection seen in old neurons may be better attributed to the Bcl/Bax ratio and appears less dependent on NF κ B translocation.

TNFR1 blocking antibody addition to either middle-age and old neurons did not change NF κ B translocation (p50 immunoreactivity) for either age. This means that TNFR2 can still

be stimulated and that TNFR2 does not mediate NF κ B activation in either age neuron. However, when TNFR2 blocking antibodies were added to middle-age neurons, NF κ B translocation was upregulated, whereas the old neurons do not show a change in NF κ B translocation. This suggests that stimulation of TNFR1 activates NF κ B, but only in the middle-age neurons. TNFR1 and its intracellular mediators may activate common pathways that lead to degradation of I κ B α , which allows translocation of NF κ B from the cytoplasm into the nucleus (Miyamoto et al., 1994; Hsu et al., 1995). In middle-age neurons, NF κ B appears to activate prosurvival pathways as demonstrated by Kaltschmidt et al. (1999). But in old neurons, the NF κ B that translocates to the nucleus fails to activate these prosurvival pathways.

Based on many other studies, the role of NF κ B in survival appears to depend on the age of neurons and the type of prior stress applied to neurons. In embryonic neurons, NF κ B translocation stimulated by TNF α promotes neuron survival (Nicholas et al., 2001; Fernyhough et al., 2005), possibly by inducing the expression of anti-apoptotic proteins such as Bcl-2 and manganese superoxide dismutase (Mattson, 2005). Also, Lu et al. (2006) showed that 10 month old p50 knockout mice had lipofuscin granule accumulation, abnormal capillaries, apoptotic glia cells, and caspase-3 positive neurons. These findings support our middle-age data that in the presence of TNF α and Abeta42, NF κ B protects neurons from cell death.

However, in embryonic hippocampal neurons, Abeta40 induced cell death by binding to TNFR1 and activating its signaling pathway which includes NF κ B translocation (Li et al., 2004). This mechanism was mediated by apoptotic protease-activating factor 1 (Apaf-1) that induced NF κ B nuclear translocation. Also, Tamatani et al. (1999) found that TNF α induces Bcl-2 expression through NF κ B activation in embryonic hippocampal neurons. TNFR1 knockouts showed downregulation of Apaf-1 activation and NF κ B nuclear translocation. Our studies show that middle-age and to a greater extent old neurons are able to translocate NF κ B in response to TNF + Abeta42, but cell death increased only for old neurons. Valerio et al. (2006) showed in postnatal cerebellar granule cells that Abeta mediated death caused NF κ B activation, which resulted in cytochrome c release and decreased neuroprotective Bcl-X_L. Inhibitors of NF κ B prevented neuronal damage, cytochrome c release, and restored Bcl-X_L levels. Toliver-Kinsky et al. (1997) found an age-related up-regulation of NF κ B in 30 month old rats that resulted in decreased cholinergic activity that could contribute to age-related cognitive deficits.

Failure of NF κ B to activate prosurvival pathways in old neurons in response to TNF + Abeta could depend on at least two factors: epigenetic regulation of these genes or redox control of activity. Whether nuclear NF κ B promotes transcription of key protective genes depends on the epigenetic state of these genes. Either critical CPG islands could be methylated or start sites blocked by histones in old neurons. According to some studies, the translocation of NF κ B in embryonic and young neurons might play an anti-apoptotic role (Guo et al., 1998; Kaltschmidt et al., 1999) or an apoptotic role (Schneider et al., 1999; Li et al., 2004) as in old neurons.

The second possible explanation for failure of NF κ B to activate prosurvival pathways involves higher ROS production (Parihar and Brewer 2007) and an oxidized redox state in old neurons (Parihar et al., in press). According to Sulciner et al. (1996) the redox state of NF κ B could be a control mechanism that determines the level of NF κ B activation or post-translational modifications of genes activated by NF κ B (Toledano et al., 1991). The differences in the redox state of old versus middle-age neurons could result in differences in NF κ B activation, thus resulting in greater cell death for old neurons compared to middle-age neurons.

Our previous study (Patel and Brewer, in press) showed that old neurons treated with TNF α plus Abeta42 fail to upregulate TNFR1 and TNFR2 like the two-fold increase in middle-age

neurons. Old neurons failed to up-regulate TNFR1 density levels above baseline levels. This failure correlates with low survival of old neurons under the same conditions. In old neurons, this combination of R1 and R2 failure to upregulate and a loss of neuroprotective Bcl-2 appears to explain the low survival for old neurons. This supports the hypothesis that either old neurons have an imbalance of signaling from R1 and R2 and/or that nuclear translocation is ineffective in old neurons. The positive correlation between survival and Bcl-2/Bax ratio suggests that ultimately the Bcl-2/Bax ratio is more important for survival than NF κ B translocation. These studies demonstrate dramatic differences between middle-age and old neurons when age-related hormonal, inflammatory and vascular complications are controlled.

Further studies are necessary to understand age-related differences in adult neurons versus embryonic neurons and possible epigenetic controls. Aging of the brain creates deficits and/or epigenetic changes that contribute to the development of neurodegenerative disease such as Alzheimer's disease (AD). These age-related changes could be regulated alterations in gene or protein expression that are "programmed aging" or pathologic metabolic responses to generalized senescent deterioration of the brain. Age-related altered regulation could be a response to subtle early damage created by environmental factors (i.e. sedentary lifestyle, head trauma, oxidative stress). Age-related changes in response to inflammatory mediators could further contribute to disease development.

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Figure 1.

Anti-p50 subunit of NF κ B (A-D) and nuclear bisbenzamide (E-H) labeling of untreated middle-age or old neurons compared to neurons treated with the combination of 100 ng/ml TNF α and 10 μ M Abeta42. I-L shows the digitally merged image with yellow indicating overlap. Scale = 10 μ m. B) Analysis of p50 translocation to the nucleus. Middle-age and old neurons were left untreated or treated with 100 ng/ml TNF α , 10 μ M Abeta42, or 100 ng/ml TNF α plus 10 μ M Abeta42. Middle-age neurons have higher nuclear density values for untreated, TNF α 1hr, TNF α 24 hr, and Abeta42 treatments compared to old neurons. TNF α plus Abeta42 treated middle-age and old neurons have significantly higher density compared to untreated middle-

age ($p < 0.001$) and old neurons ($p < 0.001$). Results are from 3 independent experiments ($n = 73$ -150 cells, 3 animals).

Figure 2.

A) Gel-shift of p50 from nuclear extracts of middle-age and old neurons treated as indicated. The band at the top of the page is protein or nucleic acid that was left in the well.

B) Middle-age neurons show significantly greater NF κ B translocation than old neurons when treated with TNF α and TNF α plus Abeta42. Middle-age and old neurons show differences in NF κ B translocation when treated with Abeta42.

Figure 3.

Middle-age and old neuron viability after treatment with TNFR1 and TNFR2 blocking antibodies. A) Middle-age and old neurons were treated with 100 ng/ml TNF plus 10 μ M Abeta42 $\pm$ TNFR1 or TNFR2 blocking antibody. When R1 is blocked, both middle-age and old neurons show a decrease in cell death percentage, however, old neurons ($p=0.001$) show a greater decrease than middle-age neurons ($p=0.03$). When R2 is blocked again both middle ($p=0.006$) and old neurons ($p=0.05$) show a decrease in cell death percentage ($n=24-48$, 2-4 animals). B) Immunocytochemistry of p50 translocation to nucleus of middle-age and old neurons. Middle-age and old neurons were treated with 100 ng/ml TNF plus 10 μ M Abeta42 either TNFR1 or TNFR2 blocking antibody. When TNFR1 blocking antibody was added; neither middle-age and old neurons decreased nuclear p50 immunoreactivity. When TNFR2 blocking antibody was added; only middle-age neurons increased nuclear p50 immunoreactivity ($p<0.001$). Old neurons do not show a change in nuclear p50 immunoreactivity ($n=30-75$ cells, 2-3 animals). The band at the top of each well is protein plus nucleic acid that did not enter the gel.

Figure 4.

A-D) Bcl-2 immunoreactivity in middle-age and old neuron. Scale = 10 μ m E) Bcl-2 and Bax immunoreactivity for middle-age neurons. F) Bcl-2 and Bax immunoreactivity for old neurons. Rearrangement of the same data indicates in G) higher control levels of Bcl-2 immunoreactivity in old neurons compared to middle-age neurons. H) in middle-age neurons Bax immunoreactivity is higher than in old neurons for TNF treatment and TNFR1 blocking antibody with TNF + Abeta (TA). I) Survival versus Bcl-2/Bax ratio of middle-age and old neurons shows a strong correlation for all conditions except middle-age neurons untreated or treated with TNF and/or TNFR1 blocking antibody and old neurons treated with TNF alone.

Figure 5.

TNF receptor mediated signaling in middle-age and old neurons. A) Middle-age neurons treated with 100 ng/ml TNF plus 10 μ M Abeta42 show significantly lower cell death compared to old neurons. Old neurons have higher p50 translocation than middle-age neurons, which might promote survival, but old neurons have less Bcl-2 that appears to override or prevent this survival signal.