



Neuronal plasticity and stressor toxicity during aging

G.J. Brewer*

*Departments of Neurology, Medical Microbiology and Immunology, School of Medicine,
Southern Illinois University, Springfield, IL 62794-9626, USA*

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Abstract

Brain aging, Alzheimer disease and stroke share common elements of deficits in calcium regulation, declines in mitochondrial function, increases in generation of reactive oxygen species (ROS), accumulated damage from ROS and immune system dysfunction. The problem is to distinguish less significant side reactions, such as gray hair, from aspects of aging that contribute to disease. Toward establishing cause and effect relationships, a neuron cell culture system is described that allows comparisons with age under uniform environmental conditions. This neuron culture model indicates that susceptibility to death by apoptosis and consequences of the inflammatory response from β -amyloid are age-related and an inherent characteristic of the neurons. Further mechanistic investigations are possible. New therapeutic approaches are suggested that combine inhibition of calcium overloads (calcium channel blockers), reduced ROS damage (melatonin, *N*-acetyl-cysteine), and bolstered mitochondrial function and energy generation (creatine). Together with newly demonstrated capabilities for adult and aged neuron regeneration and multiplication, i.e. plasticity, these approaches offer new hope toward reversing age-related decrements and damage from neurodegenerative disease. © 2000 Elsevier Science Inc. All rights reserved.

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1. Introduction

At the cellular level, brain aging, Alzheimer disease (AD) and stroke display multiple system involvement in the development of pathology. Age-related neurodegenerative diseases not only display caspase activation associated with death by apoptosis, but also show deficits in calcium homeostasis, increased generation of reactive oxygen species (ROS), alterations in energy generation and mitochondrial dysfunction. Attempts at single-target therapeutic interventions have produced less than remarkable improvements.

* Tel.: +1-217-785-5230; fax: +1-217-524-3227.

E-mail address: gbrewer@siumed.edu (G.J. Brewer).

In the mean time, 5 million patients with AD languish for an average 7 years in an inexorable down-hill course toward death. In the face of multiple system deficits, a multi-system counterattack may be more efficacious than any single inhibitor of pathology.

The relationship of cellular changes in the aging brain to disease pathology has received less attention than the pathology itself. Some pointed questions illustrate some major gaps in our knowledge. For example, in familial cases of mutations in the APP gene, which invariably lead to AD (Murrell et al., 1991; Schellenberg et al., 1992), why is the disease not manifested until the fourth decade or older? In transgenic mouse models of human APP over-expression (Quon et al., 1991; Hsiao et al., 1995), why does not β -amyloid accumulate from conception instead of waiting for middle-age or older? And why does the incidence of stroke increase with age (Curb et al., 1996)? So, what is it about aging that contributes to the pathogenesis of neurodegenerative diseases? One could hypothesize that β -amyloid slowly accumulates in susceptible regions of the brain until a threshold of toxicity is reached. Alternatively, there could be environmental aspects of aging that leave an imprint on the genome of individual aged cells, rendering them more susceptible to stressors encountered in the course of daily living. Such a proposition may be valid for glutamate excitotoxicity as well as toxicity related to β -amyloid. In this paper, I review recent progress on the comparative effects of these two stressors on cultured neurons from old and younger brains in relation to systems with strong evidence for involvement in aging. These include calcium dysregulation, mitochondrial dysfunction, generation of ROS and their targets.

2. A neuron culture model of aging

Since aging studies are notoriously confounded by individual differences in environmental, hormonal and behavioral history, we have developed techniques that impose a uniform environment for neuron culture from any age rat brain (Brewer, 1997). The basis for culture is a serum-free medium, B27/Neurobasal developed by optimizing the concentration of 32 ingredients for survival of embryonic hippocampal neurons after 4 days in culture (Brewer et al., 1993). This neuron culture model shares some of the benefits of homogenized brain techniques of the ability to perform numerous analyses on samples from the same population under constant conditions (Table 1). It improves on slice preparations in the accessibility of electrophysiologically active neurons in a uniform environment and visualization of morphology. Most importantly, these cultured neurons retain basic electrophysiologic properties of resting membrane potential, voltage-dependent calcium channels, fast inward sodium channels, voltage-dependent potassium channels and action potentials (Evans et al., 1998; Collings et al., 1999). However, age-related changes in network properties are lost in culture.

3. Age-related killing by glutamate and β -amyloid

If neurons from older animals were more susceptible to killing by glutamate or β -amyloid, then some intrinsic, age-related aspect of each neuron would be making it

Table 1
Method used to study rat brain aging

Preparation	Advantages	Disadvantages
Homogenates: (a) Soluble (b) Mitochondria (c) Synaptosomes	Uniform, defined conditions, multivariable analysis	Damage during isolation, whole brain sample, limited function
Brain slices	Network dependence, electrophysiology	Network and experience dependence contribute to variability
Neuron culture	Uniform, defined conditions, multivariable analysis, optics, superior electrophysiology	Possible changes with redevelopment in culture

more susceptible. Alternatively, if no age-related differences were found, then factors extrinsic to neurons should be studied. With the tool of neuron culture of any age under similar conditions, we compared the time and concentration-dependent toxicity of β -amyloid and glutamate (Brewer, 1998). Fig. 1 shows that hippocampal neurons from old rats are more susceptible to β -amyloid and glutamate toxicity than young neurons, which are more susceptible than embryonic neurons. These results suggest that there are factors intrinsic to the older neurons that render them more susceptible to stressors. Since we now know that death by these agents involves several steps, we can begin to dissect which of them best correlates with age to more precisely define the mechanism or age-related toxicity (Fig. 2).

We also have a system to assay which of the major theories of aging might apply to brain aging and susceptibility to neurodegenerative diseases (Table 2). These theories arise from the mitochondrial theory of aging (Linnane et al., 1989; Wallace, 1992) or the free radical

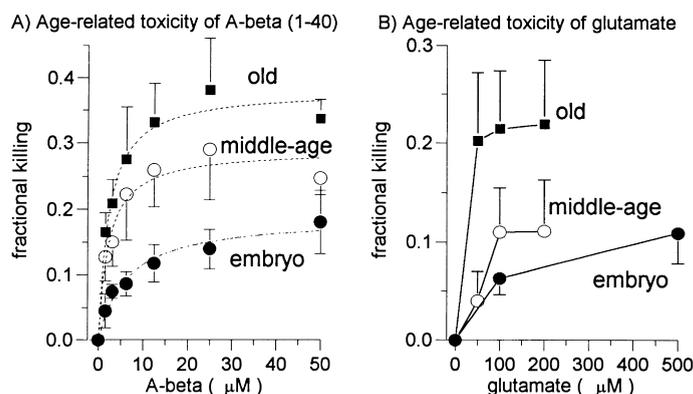


Fig. 1. Age-related killing of hippocampal neurons exposed for 24 h to (A) β -amyloid (1–40) or (B) glutamate. Neurons were isolated from old (■), middle-age (○), or embryonic (●) rats ($n = 2-4$ animals). Curves were fit to a hyperbolic single binding model (killing = $ax/(b + x)$) with $b = 0.22$ and $x = \beta$ -amyloid concentration (dashes). (B) Data are means \pm S.E. from 3 to 11 animals per age. From Brewer (1998).

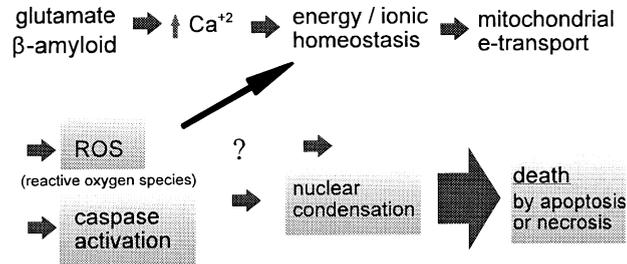


Fig. 2. Elements of age-related toxicity from β -amyloid and glutamate. Steps with a demonstrated age-related component are shaded with a box.

theory of aging (Harman, 1995) or a combined redox theory of mitochondrial aging (Ozawa, 1997).

4. Mechanisms of age-related toxicity

Fig. 2 traces some elements of a possible pathway from the point of stressor exposure (either glutamate or β -amyloid) to the end stages of death by either apoptosis or necrosis. We now ask the question, which of these steps is age-related? Working from both ends, we can ask, e.g. first whether there are age-related increases in glutamate receptor density or function that result in hyper-stimulation for the same dose of transmitter? Or, at the end, is there an age-related increase in apoptosis for neurons exposed to either glutamate or β -amyloid? By comparing responses to glutamate and β -amyloid in neurons from the hippocampus of rat embryos, middle age (10–12 months) and old (23–25 months) Fisher rats, we can determine which responses are age-related like those of neuron death. Nuclear condensation and caspase activation are aspects of apoptosis. We find age-related nuclear condensation increases from 1.5-fold for embryonic neurons exposed to 0.5 mM glutamate for 72 h to nine-fold for old neurons (Fig. 3A). Neurons treated for 24 h with 10 μ M A-beta (25–35) also show highly significant age-related increases in condensed nuclei (Fig. 3B). Similar age-related increases in caspase activation and damage from ROS were observed (Brewer et al., 2000b). These

Table 2

Aging hypotheses and the age-related pathogenesis of AD and other age-related neurodegenerative diseases

Major theories of aging	Application to Alzheimer disease
Mitochondrial decline in energy production	β -Amyloid stresses + insufficient mitochondrial energy
Loss of control of calcium regulation	β -Amyloid stress + elevated intracellular calcium
Mitochondrial generation of ROS ^a	β -Amyloid ROS + ROS from deficient mitochondria
Accumulation of damage from ROS	Buildup of β -amyloid together with damaged systems
Decline in immune system function	β -Amyloid induces uncontrolled inflammatory response
Hormonal dysregulation	β -Amyloid stress + insufficient stress response
Loss of regenerative ability	Loss of plasticity, loss of trophic responses

^a ROS = reactive oxygen species (and reactive nitrogen species); note that other neurodegenerative diseases can be thought of in terms of substitution of another stressor such as glutamate for β -amyloid.

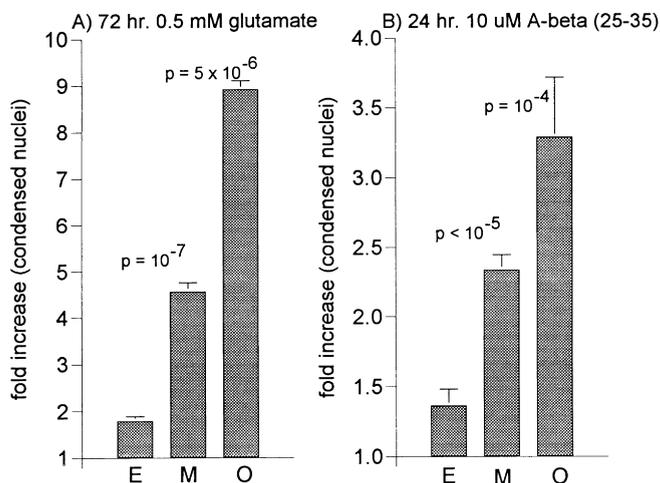


Fig. 3. Nuclear condensation in neurons cultured from embryonic (E), middle-age (M) and old age (O) rat hippocampus after exposure for (A) 72 h to 0.5 mM glutamate or (B) 24 h to 10 μ M A-beta (25–35). ANOVA for adjacent groups is shown. Means and S.E. ($n = 20$ fields of about 10 cells each). Adapted from Brewer et al. (2000b).

results suggest that age-related events upstream of activation of the caspase component of the death cascade and generation of ROS remain to be identified (Fig. 2).

At the initiation of the glutamate toxicity pathway are glutamate receptors whose expression could be age-related. Whole-cell patch clamp studies on these neurons show the largest NMDA currents for embryonic neurons, followed by old and the smallest currents in young (Cady et al., 2000). This rank order does not correlate with the age-related killing shown in Fig. 1. Therefore, NMDA receptor density is not likely to explain the increased glutamate toxicity with age.

5. Deficits in calcium regulation

A 1996 meeting of the New York Academy of Sciences cataloged abundant evidence for age and AD-related calcium dysregulation. Elements of aberrant calcium dynamic signaling and homeostasis include a pipeline from increased influx, altered responses and deficient mechanisms of efflux (Table 3). Although the calcium channel blocker, nimodipine, improved age-related changes in open-field behavior of rabbits (Deyo et al., 1989), improvement in a clinical trial for AD patients was marginal (Tollefson, 1990). Since Stout et al. (1998) have shown that inhibition of mitochondrial uptake of calcium is actually protective against glutamate toxicity, elevated cytoplasmic calcium itself is not necessarily toxic. But the combination of elevated calcium and functional mitochondria appears to promote toxicity. Since inhibition of mitochondrial function seems counterproductive to maintaining ATP levels, other approaches are needed to reduce the toxic effects of calcium on mitochondria.

Table 3
Age-related changes in brain calcium dynamic signaling and homeostasis

<i>Influx</i>	
NMDA receptor density up and GluR2 down in severe AD	Ikonomovic et al. (1999)
L voltage-gated calcium channels increase two-fold in acutely isolated medial septal neurons in rat hippocampal slices	Murchison and Griffith (1995) Thibault and Landfield (1996)
Overall 31% increase in rapid flux in rat basal forebrain neurons	Murchison and Griffith (1998)
<i>Modulators and responders</i>	
Calcium buffering:	
overall 2.5-fold decrease in capacity in rat synaptosomes	Martinez-Serrano et al. (1992)
in rat hippocampus calbindin and calretinin down 50 and 70%	
calreticulum of endoplasmic reticulum unchanged	Villa et al. (1994)
calbindin in human basal forebrain down 55%	Wu et al. (1997)
calmodulin in rat synaptic membrane-bound down 30%	Zaidi et al. (1998)
Calmodulin-dependent PKII produces NFT-like phosphorylation of tau	Singh et al. (1995)
Calcineurin phosphatase down 60% in AD	Ladner et al. (1996)
Mitochondrial storage up 70% in rat isolated basal forebrain neurons	Murchison and Griffith (1999)
Mitochondria larger and less numerous in rat cerebellum	Bertoni-Freddari et al. (1993)
Endoplasmic reticulum storage affected by β -amyloid through caspase 12	Nakagawa et al. (2000)
<i>Efflux</i>	
Na–Ca-antiporter in rat synaptic membranes unchanged	Michaelis et al. (1984)
Ca–ATPase activity in rat synaptic membranes down 47%	Zaidi et al. (1998)

6. Mitochondrial decline in energy production

Is there an energy crisis in aging and AD? Decreased cerebral blood flow and deficits in glucose uptake are frequently reported in AD (Hoyer et al., 1988). Both could be due to age-related atherosclerosis linked with the *ApoE4* allele (Kosunen et al., 1995; Small et al., 1995). In addition, PET scans using fluorodeoxyglucose show glucose hypometabolism in AD (e.g. Murphy et al., 1993). This hypometabolism is explained by regionally appropriate AD-related decrements of up to 50% in the neuronal glucose transporter 3 (Simpson et al., 1994). Normally, glucose transport is not rate-limiting for energy (ATP) generation. Indeed, the same studies that find hypometabolism in AD show no global changes in high-energy phosphates by MRI (Murphy et al., 1993). However, AD does not produce global lesions but affects single cells. These supply-side observations could actually be caused by decreased consumption of glucose from receptor down-regulation in cells with mitochondrial deficiencies.

In 1994, Flint Beal proposed that energy deficits and oxidative damage would initiate a vicious cycle of neurodegeneration. Chandrasekaran et al. (1996) proposed that the down regulation of brain oxidative phosphorylation would be expected from deafferentation. A broader review of neurodegenerative diseases by Fiskum et al. (1999) places impaired mitochondrial function at the core of elevated calcium, generation of ROS, and even increased production of β -amyloid and tau that culminate in synapse loss and neuron death. Considerable evidence has amassed to support this theory of energy, age and

Table 4
AD and age-related brain deficits in mitochondrial composition and function

<i>Age-related</i>	
Mouse electron transport complex II, III activities down 21, 41%	Kwong and Sohal (2000)
Human mitochondrial DNA mutations increase	e.g., Melov et al. (1999), Michikawa et al. (1999)
Rat NADH oxidase activity down 44% in non-synaptic mitochondria	Genova et al. (1997)
Monkey electron transport complex I, IV activities down 22%	Bowling et al. (1993)
Rat brain COX ^a activity down 25% in parietotemporal cortex	Curti et al. (1990)
<i>AD-related</i>	
COX I and IV both increase in healthy neurons, absent in neurons with tangles	Nagy et al. (1999)
COX II mRNA down 50% in hippocampus	Simonian and Hyman (1994)
Mitochondrial encoded COX I, III message down 50–65%	Chandrasekaran et al. (1994)
COX activity down 50% in isolated brain mitochondria	Parker et al. (1994)
COX activity down 25–30% in four cortical regions	Mutisya et al. (1994)
COX activity missing high affinity binding for cytochrome C	Parker and Parks (1995)
COX activity down 66% compared to citrate synthase in hippocampus	Maurer et al. (1995)
COX activity down 50–70%, but not region-specific	Wong-Riley et al. (1997)
F ₁ F ₀ ATPase reduced 50% in 6/8 cases	Schagger and Ohm (1995)
Oxygen uptake reduced 42% in biopsy homogenates	Sims et al. (1987)
Mitochondrial encoded ND4 ^a smessage down 80% in temporal cortex	Fukuyama et al. (1996)
Ketoglutarate dehydrogenase activity down 56%	Gibson et al. (2000)
Both nuclear and mitochondrial encoded subunits of ND and COX reduced	Aksenov et al. (1999)
<i>AD-related studies in culture</i>	
Uncoupling mitochondria with CCCP or azide increases β -amyloid	Gabuzda et al. (1994)
Low ATP activates PK40 which converts tau to PHF	Roder et al. (1993)
Muscle cells transfected with APP gene exhibit	
Mitochondrial inclusions and lower COX activity	Askanas et al. (1996)

^a COX, cytochrome oxidase; ND, NADH dehydrogenase.

AD-related deficits in mitochondrial composition and function (Table 4). The correlations observed in homogenates could be strengthened if measurements of mitochondrial deficits in individual live neurons were firmly linked to susceptibility of these cells to β -amyloid and glutamate.

7. Mitochondrial generation of ROS

As defects in the mitochondrial genome and nuclear mitochondrial genes accumulate with age, the generation of reactive oxygen species by mitochondria increases (Table 5). The median life span of mammals correlates with rate of mitochondrial generation of H₂O₂ and O₂⁻ and rate of respiration (Ku et al., 1993). The key question is whether stressors such as β -amyloid or glutamate initiate higher rates of ROS production in old neurons than young or whether the rate is already high in old neurons so that they are at a critical threshold for additional stress.

The other side of ROS generation is ROS protection. Do ROS detoxifying agents such

Table 5

AD and age-related changes in brain ROS and ROS defenses. (Comparisons of old rats are made to one year old rats whenever possible)

<i>AD brain</i>	
Cytoplasmic 8OHG (oxidized RNA) increased 3-fold	Nunomura et al. (1999)
Hydroxynonenal derivatives from ROS up 7-fold in hippocampus	Montine et al. (1998)
Thiobarbituric acid reactive lipid oxidation derivative up 2-fold	
Glutathione peroxidase, catalase and SOD up 30–100% in hippocampus	Lovell et al. (1995)
Thiobarbituric acid reactive lipid oxidation derivative up 2-fold, SOD down 75%	
Catalase down 35%, glutathione peroxidase unchanged in temporal cortex	Marcus et al. (1998)
Nitrotyrosine levels up 8-fold in hippocampus	Hensley et al. (1998)
Carbonyls much higher in NFT and individual neurons	Smith et al. (1998b)
 <i>ROS defenses</i>	
Thioredoxin decreases and thioredoxin reductase increase in AD	Lovell et al. (2000)
Rat brain glutathione peroxidase	
increases 2-fold	Vitorica et al. (1994)
unchanged	Rao et al. (1990), Barja et al. (1990)
Rat brain SOD	
decreased 7%, 5%	Rao et al. (1990), Semsei et al. (1991)
unchanged	Barja et al. (1990)
Cu/Zn SOD decreased 83%, Mn-SOD increased 34%	Vanella et al. (1982)
Rat brain catalase	
decreased 17%, 12%	Rao et al. (1990), Semsei et al. (1991)
unchanged	Barja et al. (1990)
Mouse brain ratio GSH/GSSG decreases 10-fold	Pallardó et al. (1998)
 <i>Transgenic mouse models over-expressing human APP</i>	
Hydroxynonenal, heme oxygenase and free iron markers of ROS increase	Smith et al. (1998a)
 <i>Cell and homogenate models</i>	
PC12 cells resistant to β -amyloid have	
(a) increased catalase and glutathione peroxidase	Sagara et al. (1996)
(b) increased endosomal/lysosomal components	Li et al. (1999)
Vitamin E protects synaptosomes from β -amyloid toxicity	Subramaniam et al. (1998)

as SOD, catalase, glutathione, thioredoxin and vitamin E change with age or AD? A recent study of lifespan in *Caenorhabditis elegans* found mutations in catalase genes blocked extension of lifespan from other mutant genes (Taub et al., 1999). These mutants such as age-1 display age-related increases in SOD and catalase (Larsen, 1993). Another worm mutation, mev-1, shortens lifespan and is associated with increased ROS from defects in succinate dehydrogenase cytochrome b (Ishii et al., 1998). If ROS increase with age, then some increase of ROS defenses is expected and observed (Table 5). Over-expression of CuZn-SOD in transgenic mice not only produces a surprise enhanced susceptibility to degeneration, but also interferes with long-term potentiation (Gahtan et al., 1998), suggesting a need for balance from catalase. Another strategy to compensate for decreased

Table 6
AD and age-related targets of ROS in the brain

<i>AD brain</i>	
Creatine kinase activity reduced by 80%, cytoplasmic bCK immunoreactivity reduced by over 90%, mitochondrial CK down 40%	Aksenov et al. (1997)
CK activity down 86%, but protein down only 14%	David et al. (1998)
α -ketoglutarate dehydrogenase reduced by 23–41%	Kish (1997)
<i>Age-related in brain</i>	
$\text{Na}^+ - \text{K}^+ - \text{ATPase}$ and $\text{Cl}^- - \text{ATPase}$ activities reduced by 60–65%	Hattori et al. (1998)
$\text{Ca}^{2+} - \text{ATPase}$ activity in rat synaptic plasma membrane down 47%	Zaidi et al. (1998)
Aconitase	Yan et al. (1997), Zheng et al. (1998)
Pyruvate dehydrogenase down 50% in dog cortex after ischemia/reperfusion	Bogaert et al. (2000)
Glutamine synthase especially sensitive	Oliver et al. (1990)
Calmodulin activity decreased by oxidation of methionines	Michaelis et al. (1996)
<i>Synaptosomes</i>	
Glucose and glutamate transporters inhibited by β -amyloid	Keller et al. (1997)

mitochondrial function with age would be to make more mitochondria. In studies of mouse brain, measures of replication of mitochondrial DNA in the face of stress are unchanged (Schmitz et al., 1999). In summary, AD and age present a clear picture of increased generation of ROS and loss of GSH, but cellular defenses present an unclear mix of insufficient elevation and loss.

8. Accumulation of damage from ROS

As the generation of ROS increases with age, deficits in cell function are proposed to accumulate until reaching a threshold above which cell death proceeds by necrosis or apoptosis (Table 6). Mecocci et al. (1993) found human cortical mtDNA accumulate 8OHdG damage at a rate 10-fold higher than nuclear DNA; the amount of mtDNA 8OHdG correlated with age ($R^2 = 0.6$) and was 15-fold higher in subjects >70 years old compared to younger ones. However, maximum levels of 8OHdG were only 0.05% of dG. The critical question is whether this amount of ROS damage contributes to neurodegenerative pathology. In order to determine this in aged neurons, correlations need to be made between 8OHdG cytoplasmic immunoreactivity and cells with activated caspases to see whether the cells that have high 8OHdG are selectively vulnerable to death by apoptosis.

Other targets with critical functions in ion, glutamate or energy homeostasis are listed in Table 6. Creatine kinase (CK) is another set of enzymes involved in cellular energetics, serving to regulate PCr levels as an energy buffer to ATP. Considering the large reductions in CK activities and immunoreactivities cited in Table 6 and since bCK mRNA was not reduced, Aksenov et al. (1997) conclude that CK activity is reduced by ROS damage, a mechanism to which CK is exquisitely sensitive (Stachowiak et al., 1998). Aksenov et al. (1998) also showed that cortical neurons treated with β -amyloid show increased levels of

immunoreactive bCK, but decreased levels of bCK activity, suggesting damage by ROS. It will be important to determine which of these enzymes or transporters is most critical in the disease process.

9. Anti-oxidant and energy-protective therapies

If generation of ROS and mitochondrial energetics are critically affected in aging so that stressors tolerated in our younger years are cytotoxic in old age, then certain anti-oxidant and energy-protective strategies may prove therapeutic. Clinical trials on AD patients have failed to show dramatic effects of either vitamin E as an anti-oxidant, or selegiline as an MAO inhibitor (Sano et al., 1997), or acetyl-carnitine to promote greater mitochondrial lipid exchange (Rai et al., 1990). Neurotrophic factors ADNF and FGF2 protect rat synaptosomes against β -amyloid and ROS toxicity acting on mitochondria and glucose and glutamate transport (Guo and Mattson, 2000), but therapeutic access of these large molecules to the brain is problematic.

Several small molecules have demonstrated in vitro efficacy against β -amyloid or glutamate toxicity with good penetration of the blood brain barrier and low side-effects. Melatonin is a naturally occurring pineal indoleamine with reduced levels with aging and AD. Melatonin (10 μ M) prevents death of neuroblastoma cells exposed to β -amyloid, reduces lipid peroxidation and eliminates the rise in intracellular calcium (Pappolla et al., 1997), as well as preventing oxidative damage to DNA (Pappolla et al., 1999). It also binds to β -amyloid and inhibits formation of toxic fibrillar beta-sheets (Pappolla et al., 1998). In addition, melatonin is effective against NMDA or hypoxia/reperfusion toxicity in cortical cultures as well as iron/ascorbate ROS damage, but did not block the elevation of calcium (Cazevielle et al., 1997). However, the efficacy of melatonin has not been studied in neurons from old animals.

Another small molecule, *N*-acetylcysteine is a precursor to glutathione that partially restored memory deficits in aged mice as well as reducing levels of lipid peroxides and protein carbonyls (Martínez et al., 2000). Part of the mechanism may involve enhanced cytochrome oxidase activity that was demonstrated in synaptic mitochondria isolated from old mice (Banachlocha, 2000). In response to glutamate toxicity, HT4 neurons were protected by *N*-acetylcysteine along with protecting against declines in glutathione (Kobayashi et al., 2000). However, *N*-acetylcysteine at 0.1 mM did not protect rat hippocampal neurons from β -amyloid (25–35) toxicity (Lockhart et al., 1994). This result contradicts numerous studies indicating a role for ROS in β -amyloid toxicity and therefore needs to be repeated at higher concentrations. *N*-acetylcysteine appears to be another small molecule with limited toxicity and demonstrated animal efficacy that should be considered for age-related neurodegenerative therapy.

By ^{31}P spectroscopy, levels of phosphocreatine (PCr) are 10% lower in mildly demented AD patients compared to controls (Pettegrew et al., 1994). Extracellular availability of creatine can increase intracellular levels of phosphocreatine, which bolsters cellular energy reserves and may protect against activation of the mitochondrial permeability transition (Hemmer and Wallimann, 1993; O’Gorman et al., 1997a). Creatine protects against glutamate and β -amyloid toxicity in cultured hippocampal neurons (Brewer and Wallimann,

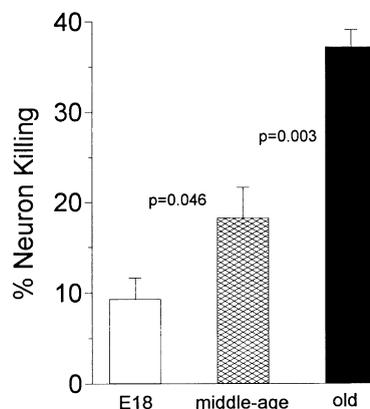


Fig. 4. Age-related killing of cortical neurons by cytokine TNF (1 $\mu\text{g/ml}$) and β -amyloid 1–40 (10 $\mu\text{g/ml}$). Neurons were isolated from frontal cortices of embryonic (E18), middle-age (11 month) and old (24 month) rats. After culture for 8 days, TNF was added, followed 24 h later by β -amyloid. After another day, neuron viability was assessed. Values are means and S.E. from isolations from 4 rats of each age. Adapted from Viel et al. (2000).

2000). Oral administration of creatine to mice results in reductions in brain lesions produced by NMDA (Malcon et al., 2000), hypoxia (Holtzman et al., 1998), and mouse models of Huntington disease (Matthews et al., 1998) and ALS (Klivenyi et al., 1999). Examination of mitochondria from biopsies of 7 AD patients showed paracrystalline inclusions (Saraiva et al., 1985). These inclusions resemble inclusions of mtCK produced in muscle cells in mitochondrial myopathy (Stadhouders et al., 1994), which can be reversed by supplementation with creatine (O’Gorman et al., 1997b). Considering the oral availability and lack of side effects, creatine should be further evaluated for AD and stroke therapy.

10. Decline in immune system function

Autoimmune diseases increase with age. Given the strong inflammatory component to AD, an uncontrolled or inappropriate response to inflammatory inducers could contribute to stressor toxicity. Alternatively, the age-related production of β -amyloid and associated neuron degeneration could produce an end-stage inflammatory response. Epidemiology suggests that use of non-steroidal anti-inflammatory agents for two or more years reduces the relative risk of AD to 0.4 (Stewart et al., 1997). In studies in cultured cells, indomethacin reduced by 50% the induction of phospholipase A by β -amyloid (25–35) (Singh et al., 1997). β -Amyloid activates brain microglia to produce TNF and other inflammatory cytokines (Meda et al., 1999). If this process is important in AD, then TNF production may increase with age or neuron killing may increase with age in the presence of β -amyloid and TNF. We find β -amyloid stimulated TNF release from microglia isolated from old rats is 50 times that from young rats (Viel et al., 2000). In addition, neurons isolated from three ages of rat cortices and cultured in the presence of 10 $\mu\text{g/ml}$ β -amyloid (1–40) with toxic levels of TNF (1 $\mu\text{g/ml}$) showed age-related death of neurons (Fig. 4). Killing in old

neurons was two-fold higher than that of middle-age neurons. These results suggest intrinsic differences in aged neurons that render them more susceptible to killing by cytokine TNF in an inflammatory response. Further investigation of the receptor and the intracellular basis for age-related changes in susceptibility may reveal novel therapeutic targets.

11. Age-related changes in synaptic plasticity and regeneration

In order to study the imprint of aging on neurons, we have introduced techniques for isolation of adult rat neurons of any age (Brewer, 1997). We fully expected the yield of neurons and their survival in culture to diminish with age. Surprisingly, neuron yield was unaffected by age out to 36-month-old rats. After 4 days in culture, survival of these old neurons was reduced by only 15% compared to neurons from middle-age rats (11 months). Survival was accompanied by the regeneration of MAP2-positive dendrites and tau-positive axons. Although neurite outgrowth was not quantified, general impressions suggest no difference in the neurite regenerative capabilities of old compared to middle-age adult neurons in culture. However, neurite outgrowth in embryonic neurons under the same culture conditions is considerably faster and more extensive. Similar techniques permit isolation, culture and regeneration of human brain surgical samples with reformation of synapses after 3 weeks (Brewer et al., 2000a). Importantly, these culture studies indicate tremendous regenerative capabilities for adult neurons of any age.

Survival of adult neurons was strongly influenced by FGF2 (bFGF) and plating density. For periods longer than 4 days and low plating densities, FGF actually stimulated division of these cells (Brewer, 1999). Neuron numbers increased 20-fold over a 6-day period. The mitotic cycle was accompanied by retraction of neurites, cell rounding, mitosis and re-extension of neurites from both daughter cells over a period of several hours. Mean doubling time was 1 day. Evidence for the neuronal nature of these cells includes MAP2, tau, neurofilament 200 and glutamate staining as well as electrophysiological properties of voltage-dependent calcium channels, fast inward sodium channels, voltage-dependent potassium channels and action potentials (Evans et al., 1998; Collings et al., 1999). The high proportion of isolated cells from the original tissue (16 000 cells/mg tissue) coupled with a high percentage of live cells that incorporate BrdU (over 50%) together argue against a progenitor population. These findings offer hope for finding conditions for regeneration of neurons in the adult brain.

Another aspect of plasticity may involve plasticity in mitochondrial function. The story emerging from ischemic preconditioning of myocardium appears to involve activation of mitochondrial K_{ATP} channels in the process of protection by a sub-lethal ischemic episode preceding a normally lethal prolonged ischemia (Ghosh et al., 2000). Activated potassium channels would depolarize the mitochondrial inner membrane and result in less mitochondrial uptake of calcium and possibly less generation of ROS from futile cycling (Richter, 1998). Such a circuit-breaker function, by action of unnatural mitochondrial uncouplers FCCP or DNP, prevents the toxicity of glutamate in cerebellar granule neurons, without blocking the rise in cytoplasmic calcium (Stout et al., 1998). Therefore, cytoplasmic calcium is not necessarily toxic, but mitochondrial function participates in toxicity. The

preconditioning phenomenon of protection has been demonstrated in cerebellar neurons pretreated with low levels of NMDA and found to be associated with autocrine production of BDNF (Marini et al., 1998), a known neuroprotective agent. The stress of exercise in rats results in elevated brain BDNF (Oliff et al., 1998). Exercise can reverse the decline in GAP-43 and synaptophysin associated with aging in mice (Chen et al., 1998). Hippocampal BDNF levels in aged rats were 2.6-fold higher than that in young rats (Kato-Semba et al., 1998), suggesting active trophic stimulation.

An early report indicated that hippocampal neurons can be protected from NMDA toxicity by potassium channel openers (Abele and Miller, 1990), but these findings have not been developed. The concept that needs exploration in neurons is that regular stressors keep mitochondria in an activatable state that can appropriately respond to increased energy demands without overproduction of ROS and/or activation of the mitochondrial permeability transition and apoptosis. The mitochondrial role in neuronal preconditioning remains to be established. Regular exercise may be the least expensive, most cost-effective treatment to reduce the pathogenesis of neurodegenerative disease.

12. Conclusions

Abundant evidence exists for deficits in calcium regulation contributing to aging as well as Alzheimer disease and stroke. The critical question is whether calcium dysregulation is merely associated with pathology, like gray hair, or is a critical cause of the pathogenesis or execution of neurodegenerative disease. The same can be said of mitochondrial function, damage from ROS, and immune system deficits. Using a neuron culture model of aging, some cause and effect relationships can be established. New therapeutic approaches are suggested that take a multisystem approach to treatment of age-related neurodegenerative disease including inhibition of calcium overloads, and ROS damage, and bolstered mitochondrial function and energy generation and exercise. Together with newly demonstrated capabilities for adult and aged neuron regeneration and multiplication, these approaches offer new hope toward reversing the damage of neurodegenerative disease among the elderly.

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