8 Membrane Cytoskeletal Protein Alterations in Alzheimer’s Disease

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ABSTRACT

Alzheimer’s disease (AD) affects millions of people worldwide, and therefore is of critical importance as a public health issue. Fundamental insight into the underlying molecular mechanisms leading to brain cell death and formation of senile plaques has recently been obtained from our laboratory [Hensley et al. (1994), Proc. Nat. Acad. Sci. USA 91:3270–3274; Butterfield et al. (1994), Biochem. Biophys. Res. Commun. 200:7100–7115]. Previous research employing electron paramagnetic resonance spectroscopy described alterations in the physical state of cytoskeletal proteins in non-neuronal erythrocyte membranes in AD, pharmacologic modulation of cytoskeletal protein-protein interactions by promising AD therapeutic agents, and choline transport anomalies in AD red cells. This paper reviews these findings of membrane alterations related to AD.

INTRODUCTION

Alzheimer’s disease (AD), the major dementing disorder of the elderly, affects over 4 million people in the United States, where it is the fourth leading cause of death.¹ The prevalence of AD increases exponentially with age, and it is estimated that over 10 million person will be diagnosed with AD shortly after the turn of the century.¹ Consequently, AD is a
major public health problem of great importance to victims, their families, to health-care providers, and to fiscal planners.

Clinically, AD is characterized by profound memory loss and decline in other cognitive functions. Pathologically, two characteristic hallmarks of this disease are seen on autopsy: (a) senile plaques (SP), composed of a core of aggregated β-amyloid surrounded by dystrophic neurites and other cellular components; and (b) neurofibrillary tangles (NFT), composed of paired helical filaments of aggregated cytoskeletal proteins, principally tau, a hyperphosphorylated protein in AD.1-3

The etiology and pathogenesis of AD remain unexplained. Various hypotheses have been previously offered and include: Trace metal imbalance, viral infection, metabolic aberrations, and genetic abnormalities.1 Any hypothesis must account for the age-dependence of

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**Fig. 8.1** “Molecular shrapnel” model for β-amyloid peptide free radical-induced aggregation and neurotoxicity.
onset for AD, and must account for the large number of different abnormalities in membrane enzymes, transport proteins, and lipids reported in AD.\textsuperscript{1-2} Recently, we proposed a unifying hypothesis for the pathogenesis of AD involving the production of highly-reactive peptide free radicals from β-amloid (Aβ).\textsuperscript{4} Figure 8.1 shows the main tenets of our findings. β-amloid, which is produced in AD by alternate processing of the transmembrane protein, amyloid precursor protein (APP), in an oxygen-dependent process spontaneously forms free radicals that we detected by electron paramagnetic resonance (EPR) spin trapping techniques and HPLC-mass spectrometry.\textsuperscript{4} We subsequently showed that the "molecular shrapnel" of highly-reactive free radicals were able to reduce the paramagnetism of a lipid-specific spin label deep within the lipid bilayer of neocortical synaptosomal membranes,\textsuperscript{5} a prediction of the model developed. The Aβ-derived peptide free radicals could rapidly bind to vulnerable enzyme and transport proteins, compromising their function and leading to cell death.\textsuperscript{4-6} The Ca\textsuperscript{2+}-transporter and Ca\textsuperscript{2+}-ATPase are known to be affected by Aβ,\textsuperscript{7} and intracellular increases in this divalent cation could lead to cellular processes that lead to the death of neurons and other brain cells.\textsuperscript{4-6}

APP is a protein found in several cell types including platelets.\textsuperscript{8} Consequently, APP could be processed in peripheral tissue leading to full-length β-amyloid extracellularly. If processes similar to those found by us\textsuperscript{4-6} in brain cells were to take place in peripheral tissue, then perhaps the myriad of reports of enzymatic, transporter, and cytoskeletal protein abnormalities in AD could be explicable.\textsuperscript{9-11} This paper reviews the research from our laboratory that shows membrane abnormalities in non-neuronal tissue in AD.

\textbf{RESULTS AND DISCUSSION}

\textit{Spin Labeling Methods}

Spin labeling is an EPR method that has provided considerable insight into the structure and function of cell membranes.\textsuperscript{9,10,12-18} Spin labels, usually of the nitrooxide type, are paramagnetic molecules whose structure results in a covalent attachment to or physical relationship with
biological molecules like those found in membranes. Figure 8.2 gives the structure of spin labels used in the studies from our laboratory.

MAL-6 and MTS are protein-specific spin labels\(^{19-28}\); Tempamine can be selectively coupled to sialic acid or terminal galactose residues as desired\(^{29,30}\), and 5-NS, 12-NS, and CAT-16 provide information on the

![Structures of spin labels](image)

Fig. 8.2 Structures of spin labels used in our studies on Alzheimer's disease.
physical state of the lipid bilayer.\textsuperscript{5,16–18,31–33} The theory of spin labeling is beyond the scope of this review; however, detailed explanations of this method and its applications to membrane studies are available.\textsuperscript{12–18} The EPR spectra of spin labels bound to membrane components are exquisitely sensitive to molecular motion on the time scale associated with the movement of membrane protein and lipid components. Further, spin label spectra give insight into the polarity of the local microenvironment of the spin label. Finally, generally the spin label is the only paramagnetic species in the biological membrane; consequently, the EPR spectrum reflects the local microenvironment of the paramagnetic center of the spin label. For example, the lipid-specific

![Diagram](image)

**Fig. 8.3** (Top): Schematic diagram showing weakly- (a) and strongly- (b)- immobilized covalent SH binding sites on membrane proteins. (Bottom): Typical EPR spectrum of the low-field ($M_t = +1$) lines of MAL-6 bound to membrane proteins in erythrocyte membranes.
spin labels, 5-NS and 12-NS, give physical information about the lipid bilayer near the lipid-water interface and the middle of the bilayer, respectively.

The protein-specific spin label MAL-6 binds to cytoskeletal proteins in erythrocyte membranes, and based on selective protein isolation studies and antibodies directed against the spin label, most of the spin label is bound to spectrin, the chief cytoskeletal protein. A typical spectrum of MAL-6 bound to cytoskeletal proteins in erythrocyte membranes is shown in Fig. 8.3. It is seen that at least two kinds of spin label reaction sites are found: Those in which MAL-6 has relatively free rotation (weakly-immobilized sites, W) and those in which the motion of the spin label is highly restricted (strongly-immobilized sites, S). The EPR spectral ratio of the \( M_x = +1 \) low-field line W/S (Fig. 8.3) is a highly sensitive and convenient measure of the physical state of cytoskeletal proteins. Procedures and agents which decrease cytoskeletal protein-protein interactions lead to increased motion of the spin label and consequently to increased W/S ratios of MAL-6. In contrast, when cytoskeletal protein-protein interactions are increased, for example by cross linking spectrin to the major transmembrane protein band 3, the motion of MAL-6 decreases and the W/S ratio decreases.

**Membrane Alterations in AD Erythrocyte Membranes**

Our laboratory was the first to suggest that AD should be considered a disorder associated with a membrane defect. EPR spin labeling methods were used to investigate the physical state of membrane cytoskeletal proteins and bilayer lipids in erythrocyte ghosts in AD and aged-matched controls. The physical state of membrane cytoskeletal proteins from AD erythrocytes was altered in 11 of 15 AD subjects examined relative to control membranes (\( P < 0.025 \)). However, using 5-NS no change in the physical state of the lipid bilayer near the lipid-water interface could be demonstrated. Consistent with this finding, we recently showed that \( \beta \)-amyloid-derived peptide free radicals did not affect the 5-NS spin label in neocortical synaptosomal membranes; however, the 12-NS was greatly affected by these free radicals showing a membrane regional vulnerability to peptide free radical damage to membranes.
Since we showed that the physical state of erythrocyte membrane cytoskeletal proteins was altered in AD\textsuperscript{35} and we had reported that alterations in the physical state of cytoskeletal proteins could affect the physical state of cell-surface carbohydrates and vice versa,\textsuperscript{18,24} we examined the physical state of cell-surface carbohydrates using selective spin labeling procedures for sialic acid and terminal galactose that we developed in our laboratory.\textsuperscript{29,30} Using the Tempamine spin label covalently attached to cell-surface glycoconjugates, we were unable to detect any change in the physical state of the external cell-surface in AD erythrocytes,\textsuperscript{36} again consistent with our recent studies with synaptosomal membranes.\textsuperscript{5}

Biochemically, there is a major cholinergic deficit in AD brain\textsuperscript{1,37}: Choline acetyltransferase (CAT) activity is decreased by as much as 90 percent and levels of the neurotransmitter, acetylcholine (ACh), are also decreased.\textsuperscript{38} To test the hypothesis that one way CAT activity could be decreased is by loss of substrate, we measured the efflux of choline from erythrocytes. The efflux rate from AD membranes was considerably greater than those from age-matched controls [efflux rate constant (SEM), N=8: AD, 0.0172 (0.0021) min\textsuperscript{-1}; control, 0.0136 (0.0024) min\textsuperscript{-1}, P < 0.01], consistent with the hypothesis of an altered choline transport system. Modification of the external portions of transmembrane proteins in erythrocyte membranes produced no change in choline efflux,\textsuperscript{39} suggesting that choline efflux may be affected by interactions on the cytoplasmic side of the membrane and consistent with the previous EPR studies.\textsuperscript{35,36} Fodrin, a cytoskeletal protein in brain with the same 106-amino acid repeat sequence as spectrin, has been implicated in the mechanisms of memory,\textsuperscript{40} leading to the speculation that an altered cytoskeletal protein network in AD could be associated with one of the clinical hallmarks of this disorder. Another extraneuronal transport system has been reported altered in AD: The Li\textsuperscript{+}/Na\textsuperscript{+} countertransporter in AD erythrocytes.\textsuperscript{41} That two different transport systems are altered in AD erythrocyte membranes is consistent with either an altered cytoskeletal network of proteins\textsuperscript{35} that interacts with the cytoplasmic pole of transmembrane transport proteins\textsuperscript{18,24} or altered transporters as a consequence of peptide free radical modulation of function.\textsuperscript{4-6} These two alternatives need not be mutually exclusive.
Pharmacologic Modulation of Cytoskeletal Protein-Protein Interactions

Effective therapeutic intervention in AD still is an important, but as yet, elusive goal. Since the levels of ACh are greatly diminished in AD and cholinergic neurons are lost in this disorder, therapeutic strategies have been aimed at reversing the loss of ACh. Among these strategies have been precursor loading, use of cholinergic muscarinic agonists, and the use of acetylcholinesterase (AChE) inhibitors. Precursor loading has in general not been successful in AD. While directly-acting ACh agonists have the advantage of binding to muscarinic receptors that are relatively spared in AD, these agents are generally short acting and many do not cross the blood-brain barrier. AChE inhibitors have been used to increase availability of ACh. However, not all AChE inhibitors are effective in AD, suggesting that mechanisms in addition to simple AChE inhibition may be operative in the use of the effective agents.

Figure 8.4 gives the structure of several of the agents used in studies in our laboratory. Each of these agents leads to increased levels of ACh.

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine), sometimes referred to as THA, is an AChE inhibitor that is effective in halting the cognitive decline in a subset of AD patients, but is ineffective in others and can lead to serious hepatotoxicity. A host of pharmacologic effects of tacrine have been reported, including direct effect on muscarinic and nicotinic receptors, inhibition of carbachol-stimulated phosphatidylinositol hydrolysis, inhibition of monoamine oxidase, blockade of dopamine and serotonin uptake, and modulation of voltage-gated K+ channels. Since not all AChE inhibitors are effective in AD, we suggested that in addition to inhibition of AChE, these agents may be affecting the physical state of cytoskeletal proteins, which through their interactions with transmembrane proteins, could affect multiple pharmacologic functions. Consistent with this hypothesis, tacrine highly significantly decreased the W/S ratio of MAL-6 in erythrocyte membranes [approximately 55% decreased from control, P < 0.00001], suggesting that this agent greatly increased cytoskeletal protein-protein interactions. A similar decline in the W/S ratio of MAL-6 in neocortical synaptosomal membranes (P < 0.001) was also observed by us. Other anti-AChE compounds did not induce this effect, nor was the presence of negatively charged sialic acid on the cell surface
Fig. 8.4 Structures of the potential AD therapeutic agents used in our studies.

required. The physical state of the lipid bilayer was not affected by tacrine. These results are consistent with the hypothesis that this agent interacts with cytoskeletal proteins in erythrocyte and neocortical
synaptosomal membranes. Structure-activity studies of tacrine\(^{46}\) indicated that all three rings and the amino group in the 9-position were required to induce the observed effect. We synthesized the N-methylacridinium salt (NMAMS, Fig. 8.4) in order to determine if the positive charge was necessarily required in the 9-position or whether a positive charge para to the 9-position would serve just as well.\(^{47}\) The results indicated that with NMAMS, a decreased W/S ratio was found, but considerably less active than tacrine, suggesting that there exists a structural component to the interaction of tacrine with cytoskeletal proteins.\(^{47}\)

Velnacrine, the 1-OH derivative and chief metabolite of tacrine, is proposed as an alternative to tacrine to slow the cognitive decline of AD patients.\(^{44}\) The 1-OH group was proposed to serve as a site for glucoronidation, thereby making velnacrine more water soluble and less hepatotoxic than tacrine.\(^{44}\) EPR spin labeling studies showed that in both erythrocyte\(^{47}\) and neocortical synaptosomal\(^{48}\) membranes, velnacrine deceased the W/S ratio of MAL-6 about half the degree that tacrine did (Table 8.1). Since velnacrine is considerably less hepatotoxic than tacrine\(^{52}\) and the maximal dosage of the former is 1.5 times greater than the latter,\(^{53}\) it is interesting to speculate that if the same smaller change in cytoskeletal protein-protein interactions were to occur in hepatocytes, then the decreased hepatotoxicity of velnacrine relative to tacrine might be explained.

**Table 8.1 Relative Effects of Potential Alzheimer’s Disease Therapeutic Agents in the W/S Ratio of MAL-6 in Neocortical Synaptosomal Membranes to That in Erythrocyte Membranes**

<table>
<thead>
<tr>
<th>Agent</th>
<th>(W/S)(^b)(_{RBC})</th>
<th>(W/S)(^b)(_{synap})</th>
<th>Ratio(^c) synap/RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrine</td>
<td>55%</td>
<td>20%</td>
<td>0.44</td>
</tr>
<tr>
<td>Velnacrine</td>
<td>30%</td>
<td>10%</td>
<td>0.33</td>
</tr>
<tr>
<td>HP749</td>
<td>20%</td>
<td>5%</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(a\) 1.5–1.6 mM for each agent.

\(b\) Percent decrease from the respective control.

\(c\) Ratio of percent decrease from the respective control in the two systems.
The failure of cholinergic therapy alone to enhance mentation in a larger pool of AD patients\(^49\) attests to the molecular complexity of this disorder. Neuropathological studies of autopsied AD brains indicate a deficiency in the levels of other neurotransmitters, notably the catecholamine norepinephrine.\(^1\) Thus, true efficacy in AD therapy necessitates an agent which combats the dysfunctions of multiple neurotransmitter systems. Initial \textit{in vivo} and \textit{in vitro} studies of N-(n-propyl)-N-(4-pyridinyl)-1H-indol-1-amine (HP749, Fig. 8.4), a compound that demonstrates both noradrenergic and cholinomimetic properties, appear promising.\(^53,54\) Although not an AChE inhibitor, HP749 exhibits cholinomimetic properties that do not stem from direct agonist activity. In rats, HP749 also exhibits noradrenergic properties characteristic of anti-depressants. We used EPR spin labeling with MAL-6 to assess whether this agent, like tacrine and velnacrine, would inhibit the motion of this protein-specific spin label bound to the cytoskeletal protein network in erythrocyte and synaptosomal membranes. At the same concentration at which tacrine caused a 55\% reduction of the W/S ratio in erythrocyte membranes, HP749 caused a 20 percent decrease (P < 0.00002, N=6).\(^55\) Similarly, in synaptosomal membranes, whereas tacrine caused a 20\% decrease in the W/S ratio of MAL-6, HP749 caused a 5\% decrease (P < 0.0007, N=6).\(^55\) We conclude that HP749 affects the physical state of cytoskeletal proteins in both erythrocyte and synaptosomal membranes in the same trend as tacrine and velnacrine, but to a lesser extent (Table 8.1). It is conceivable that such alterations could account for multiple neurotransmitter system effects of HP749.\(^53,54\) Given that velnacrine is less toxic than tacrine and the effect on cytoskeletal proteins of HP749 is less than that of velnacrine, these EPR studies are consistent with the speculation that HP749 may be even less toxic to the liver than velnacrine.

Not an AChE inhibitor, the ability of HP749 to induce conformational changes in cytoskeletal proteins in the same trend as tacrine supports the hypothesis noted above that a mechanism in addition to AChE inhibition may be partially responsible for the improved mentation in some AD patients treated with tacrine.

Acetylcarnitine (ALC) also leads to increased levels of ACh in brain, but in contrast to tacrine and velnacrine, by a synthetic pathway coupling CAT with carnitine acetyltransferase.\(^57\) ALC is a natural metabolite, and
it is reported that large doses of this compound can be given to people with no obvious clinical contraindications. Clinical trials with ALC show great promise of significantly delaying the cognitive decline of AD patients with no reported liver toxicity. We used MAL-6 to investigate the physical state of cytoskeletal proteins in erythrocyte membranes upon treatment with ALC. Relative to the control W/S mean value, a highly significant, but small, decline in the W/S ratio of MAL-6 was found (approximately 10% decreased value, P < 0.001). This small change coupled with the large doses that can be given is consistent with the notion that ACh levels in brain can be increased but with no or minimal liver damage. Palmitoylcarnitine was unable to elicit any change in the physical state of cytoskeletal proteins, suggesting that the acetyl group is not hydrophobic enough to direct ALC to the lipid bilayer, and that the observed effects on cytoskeletal proteins are due to direct interactions of the agent with this region of the membrane.

Our recent findings of β-amyloid-derived peptide free radical involvement with membrane damage in brain membranes suggests another approach to therapeutic intervention in AD: Appropriate brain accessible anti-oxidant compounds. Our initial studies with these agents are very promising. The loss of paramagnetism of the 12-NS lipid specific spin label can be completely inhibited with appropriate anti-oxidants. Continued efforts on this line of research are in progress in our laboratory.

CONCLUSIONS

EPR spin labeling has provided considerable insight into the physical state of neuronal and non-neuronal membranes in AD, and the pharmacological modulation of the membrane. Our recent formulation of a unifying hypothesis for the pathogenesis of AD involving β-amyloid-derived peptide free radicals that accounts for the age-dependence of and the myriad of reported membrane abnormalities in AD, as well as the formation of senile plaques, suggests additional directions for study of this important disorder. EPR offers great advantages for these studies that are currently in progress in our laboratory.
ACKNOWLEDGMENTS

The research skills of and discussions with the following people are gratefully acknowledged: Donna Palmeri, Anu Rangachari, Sandra Umhauer, Nathan Hall, Kenneth Hensley, Polly Shrewsbury, Beverly Howard, and John Carney. These studies were supported in part by grants from the National Science Foundation (EHR-9108764; CTS-9307518) and the National Institutes of Health (AG-10836). Hoechst-Roussel Pharmaceutical Corporation is gratefully acknowledged for donations of velnacrine and HP-749.

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