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# Age-associated changes in synaptic lipid raft proteins revealed by two-dimensional fluorescence difference gel electrophoresis

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**Abstract**

Brain aging is associated with a progressive decline in cognitive function though the molecular mechanisms remain unknown. Functional changes in brain neurons could be due to age-related alterations in levels of specific proteins critical for information processing. Specialized membrane microdomains known as 'lipid rafts' contain protein complexes involved in many signal transduction processes. This study was undertaken to determine if two-dimensional fluorescence difference gel electrophoresis (2D DIGE) analysis of proteins in synaptic membrane lipid rafts revealed age-dependent alterations in levels of raft proteins. Five pairs of young and aged rat synaptic membrane rafts were subjected to DIGE separation, followed by image analysis and identification of significantly altered proteins. Of 1046 matched spots on DIGE gels, 94 showed statistically significant differences in levels between old and young rafts, and 87 of these were decreased in aged rafts. The 41 most significantly altered ($p < 0.03$) proteins included several synaptic proteins involved in energy metabolism, redox homeostasis, and cytoskeletal structure. This may indicate a disruption in bioenergetic balance and redox homeostasis in synaptic rafts with brain aging. Differential levels of representative identified proteins were confirmed by immunoblot analysis. Our findings provide novel pathways in investigations of mechanisms that may contribute to altered neuronal function in aging brain.


*Keywords:* 2D DIGE; Brain aging; Energy metabolism; Lipid rafts; Synaptic dysfunction

## 1. Introduction

Prominent features of brain aging are the progressive decline in cognitive function and impairment in learning and memory formation (Albers and Beal, 2000). However, the mechanisms underlying age-associated changes in neuronal function remain undefined. Of theories proposed to explain the phenotypes observed in aging, the most widely accepted ones include enhanced oxidative stress (Floyd and Hensley, 2002), mitochondrial dysfunction (Albers and Beal, 2000), $Ca^{2+}$ dyshomeostasis (Khachaturian, 1994), synaptic atrophy (Lee et al., 2000), cytoskeletal abnormalities (Lee et al., 2000), and glial activation (Nichols, 1999). Oxidative stress accompanied by compromised energy metabolism and excessive production of reactive oxygen species (ROS) appears to play a central role in aging. This is supported by the observed decrease in respiratory function and oxidative damage to cellular DNA, proteins and lipids (Albers and Beal, 2000).

It is now clear that there is a loss of ~10–15% of synaptic junctions in aged brain as measured by microdensitometry (Masliah et al., 2006). Synaptic plasma membrane (SPM) preparations isolated from brain contain the synaptic junctions of neurons and offer excellent material for the

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studies of synaptic protein organization in specific domains, including specialized microdomains enriched in cholesterol, sphingolipids, and signaling proteins. These microdomains, termed 'lipid rafts', appear to serve as local organizing sites for the coordination of numerous signaling events by bringing receptors, effectors, and downstream signaling proteins into close proximity and promoting interactions (Simons and Toomre, 2000). It is important to note that the synaptic localization of proteins that affect neuronal plasticity and cognition, the glutamate receptors, is determined by the positioning of lipid raft domains at synapses (Hering et al., 2003; Hou et al., 2008). Given that lipid rafts provide platforms for a diverse array of neuronal processes, identification of age-dependent changes in the protein composition of these microdomains will likely provide insights into the molecular basis for impaired neuronal function with increasing age. The goal of this study was to determine if a proteomic analysis revealed any consistent differences in expression of proteins in lipid rafts isolated from SPMs of young *vs.* aged rat brains. We used two-dimensional fluorescence difference gel electrophoresis (2D DIGE) to assess age-related changes in the synaptic lipid raft compositions. Many proteomic strategies have been applied to studies of the brain (Rohlff, 2000), particularly in neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, as well as schizophrenia (Rohlff, 2000; Jiang et al., 2003). Difference gel electrophoresis is designed to eliminate inter-gel variability apparent in traditional two-dimensional gel electrophoresis (2DE) and improve reproducibility by allowing co-electrophoresis of up to three different samples in a single gel (Alban et al., 2003). This strategy improves the quantification of differential expression in comparative proteomics.

Results of our analyses comparing five pairs of young *vs.* aged SPM rafts revealed significant changes in synaptic lipid raft proteins with increasing age. Forty-one raft proteins showing the most significant differences between raft domains from young and old were identified. A substantial number of the identified proteins are associated with energy metabolism. Many of those proteins may be components of the plasma membrane redox system (PMRS) (Ly and Lawen, 2003) and play important roles in energy regulation and maintenance of redox homeostasis. Our observations suggest that disruption in both bioenergetic and redox balance in lipid domains may contribute to altered neuronal function observed in aged brain.

## 2. Experimental procedures

### 2.1. Materials

Sources for the various primary antibodies were as follows: anti-Flotillin-1 (FLT-1) and anti-Thy-1, BD Biosciences; anti-$Na^+/K^+$-ATPase, anti-ATP synthase beta subunit (ATPB), and anti-voltage-dependent anion-selective channel protein 1 (VDAC1), Affinity Bioreagents; anti-glial fibrillary acidic protein (GFAP), Chemicon International; anti-MnSOD, Stressgen; anti-mitofilin (IMMT), Proteintech Group; anti-NADH dehydrogenase (ubiquinone) Fe-S protein 3 (NDUFS3), MitoSciences; anti-cytochrome *c*, Santa Cruz Biotechnology; and anti-glutamate dehydrogenase (GDH), Novus Biologicals. Immobilized pH gradient (IPG) strips, Pharmalyte, urea and CyDye DIGE fluor minimal labeling kit were purchased from GE Healthcare. Alkaline phosphatase-conjugated secondary antibodies, ficoll, CHAPS, thiourea, iodoacetamide, trifluoroacetic acid (TFA), dimethylformamide (DMF), α-cyano-4-hydroxycinnamic acid, and horseradish peroxidase-coupled cholera toxin subunit B (CTXB) were from Sigma. Acetonitrile and dithioerythritol (DTT) were obtained from Fisher Scientific International. Amplex Red cholesterol assay kit and Sypro Ruby were from Invitrogen, Bicinchoninic Acid protein assay reagents from Pierce Biotechnology, PVDF membranes and ZipTips from Millipore, trypsin from Promega, and protease inhibitor cocktail from Calbiochem.

### 2.2. Animals

Eight pairs of Fisher 344/Brown Norway hybrid (F344/BNF1) rats at 5 months (young) and 34 months of age (old) (five pairs for Cy dye labeling and three for immunoblot analysis) were obtained from an NIA colony maintained by Harlan Industries (Indianapolis, IN, USA). All protocols were implemented in accordance with NIH guidelines and approval by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

### 2.3. Isolation of SPMs, mitochondria and SPM rafts from young and old rat brains

Fisher 344/BNF1 rats were anesthetized by $CO_2$ inhalation according to the IACUC guidelines. Rats were decapitated using a guillotine and brains removed quickly. The brains from one young and one old rat were processed in parallel. Each whole brain was homogenized and processed immediately for the isolation of synaptosomes and mitochondria by discontinuous ficoll density gradient centrifugation as previously described (Michaelis et al., 1983). Synaptosomes were lysed with a hypotonic buffer (3 mM Tris–HCl, pH 8.0, with a cocktail of protease inhibitors) and centrifuged at 64,200 × *g* for 15 min in a Ti 60 rotor (Beckman Coulter) to isolate the SPMs. The SPM and mitochondrial pellets were both homogenized in buffer containing 10 mM Tris–HCl, 50 μM $MgCl_2$ and 0.32 M sucrose, pH 7.4. Eight pairs of lipid raft preparations were isolated from SPMs using discontinuous sucrose density gradient centrifugation (Jiang et al., 2007). In brief, the SPMs from each animal were solubilized in an equal volume of solubilization buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5, containing 2% Brij 98), to achieve 1% Brij 98 as the final concentration, and incubated on ice for 30 min. The suspension was mixed 1:1