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# The Application of Proteomics and Genomics to the Study of Age-Related Neurodegeneration and Neuroprotection

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## ABSTRACT

**The present study aimed to acquire more information on aging-related alterations, using proteomic and genomic analyses of hippocampus from young (8 months) and old (27 months) rats. In the old rats, the proteomic analysis identified changes in proteins related to the iron-mediated oxidative stress (OS) pathway, including reduction in antioxidant enzymes (*e.g.*, peroxiredoxin, cytochrome c oxidase) and induction of ferritin. Furthermore, the neurofilament light peptide, associated with neurodegenerative processes, was enhanced and binding/chaperone proteins were altered in old vs. young rats. At the genes levels, significant molecular changes related to neurodegeneration were identified in aged rat hippocampus. Thus, the effects of the potent neuroprotective compounds, the anti-Parkinson drug, rasagiline and the anti-Alzheimer drug, ladostigil (1 mg/kg, for 30 days) on gene expression in the hippocampus were further investigated. Both drugs reversed the effect of aging on the expression of various mitochondrial and key regulator genes involved in neurodegeneration, cell survival, synaptogenesis, oxidation, and metabolism. These results support the hypothesis that OS and mitochondrial dysfunction may play a pivotal role in aging and age-associated neurodegenerative diseases, and can serve as potential clinical targets for future therapy.** *Antioxid. Redox Signal* 9, 169–179.

## INTRODUCTION

AGING IS CHARACTERIZED BY decrements in tissue function and accumulation of mitochondrial DNA mutations, particularly in the brain that contains postmitotic cells (88). Brain aging comprises multifactorial processes and affects by alterations in many gene/protein expressions involved in various biochemical and signaling pathways (62). Thus, proteomic and genomic strategies provide a powerful approach for addressing the complexity of brain function and age-related neurodegenerative diseases, (5, 6, 16, 45, 47, 56, 58, 64, 66). To date, gene and protein expression profiles have been used separately to study aging and/or age-related neurodegenerative disorders in the central nervous system in animal models (8, 14, 20, 54, 55, 69, 70) and human postmortem tissues (7, 24, 36, 46, 48, 74). Indeed, measurements of multiple changes in the RNA transcription arrays provide an excellent tool for screening complex processes, without preliminary assumption on the contribution of specific genes (15). Another approach is proteomic analysis, which can be an ideal tool to elucidate translational and post-translational modifications to detect native protein alterations. Thus, the combination of transcriptomic tools and proteomic technology provide more comprehensive overview of the interplay among aging processes and the context in which a specific molecule or pathway may be operating (41, 58).

Many lines of evidence suggest that mitochondrial dysfunction and oxidative stress (OS) play an important role in age-related neurodegenerative diseases (21, 22, 29). It is well established that OS increases in the aging brain (57), and iron-mediated OS results in reactive oxygen species (ROS) generation, inflammation, cognitive decline, and neuronal loss in aging and age-associated disorders, including Alzheimer's disease (AD), Parkinson disease (PD), and Huntington's diseases

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(HD). Thus, the oxidative mechanisms responsible for ROS-mediated cell injury involve mainly peroxidation of lipid membranes, DNA and protein oxidation, and subsequent neuronal cell death that contributes to disease pathogenesis (30, 51). A previous report described a decrease in the activity of the enzyme catalase, that catalyzes the breakdown of hydrogen peroxide ($H_2O_2$), within the parietal-temporal cortex and basal ganglia, as determined from postmortem AD brain tissues (25). Furthermore, an increase in monoamine oxidase (MAO)-B activity within reactive microglia in AD and PD brain tissues is considered to contribute high levels of $H_2O_2$ formation, as a by-product of amine turnover (65). Failure to remove excess $H_2O_2$ as an outcome of reduced glutathione (GSH) (61) and catalase activity, may result in the generation of reactive free oxygen and $OH^-$, produced through the interaction of $H_2O_2$ with chelatable (free ionic) iron (Fenton chemistry), and would initiate OS processes (87). Indeed, high concentrations of reactive iron can increase OS-induced neuronal vulnerability and enhance the toxicity of environmental or endogenous toxins (85).

The present study describes significant molecular changes in aged rat hippocampus at the protein and transcriptional levels associated with OS, mitochondrial dysfunction, and neurodegenerative diseases. In addition, the effects of our two potent neuroprotective compounds, the anti-Parkinson drug/MAO-B inhibitor, rasagiline (86), and the anti-Alzheimer drug, ladostigil (TV3326) (78), on gene expressions in the hippocampus of young and old rats were investigated. Our previous studies (1, 63, 76) demonstrated that both drugs exert neuroprotective/neurorescue activities via multiple survival pathways, including the stimulation of protein kinase C (PKC) pathway, activation of the Ras–phosphatidylinositol 3-kinase (PI3K)–Akt pathway, and regulation of several mitochondrial genes and proteins related to the Bcl-2 family members, in various in vivo and in vitro models of PD and AD. Additionally, rasagiline was previously shown to prevent the fall in mitochondrial membrane potential ($\Delta\Psi m$) and the opening of mitochondrial voltage-dependent anion channel (43), and to upregulate antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione (9, 42). Complementing the valuable information generated through genomic techniques with the integrative knowledge of protein expression will enable us to develop more efficient diagnostic markers and neuropharmacological agents to treat brain diseases. Our study provides a basis for formulation of future hypothesis-driven experiments to determine neuroprotective mechanism of action of anti-age-associated disease drugs.

## MATERIAL AND METHODS

### *Animal protocol and drug treatment*

All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Technion Animal Ethics Committee, Haifa, Israel. Female Wistar rats (Harlan, Jerusalem, Israel), young animals of 8-months-old ($n$ = 15), weighing 360–420 g, and aged animals of 27-months-old ($n$ = 15), weighing 480–530 g, were sacrificed by decapitation. Thirty days prior to decapitation, young ($n$ = 4) and old rats ($n$ = 4) were treated by gavage with the anti-Parkinson drug, rasagiline [Agilect®) ($N$-propargy-1R-aminoindan)] (0.1 mg/kg per day), or the anti-Alzheimer's drug, ladostigil (TV3326) [($N$-propargyl-(3R) aminoindan-5yl)-ethyl methyl carbamate] (1 mg/kg per day) or vehicle (sterile saline, control group) for 30 days. The drugs, rasagiline and ladostigil, were kindly provided by Teva Pharmaceutical Co. (Netanya, Israel). The brains were dissected for the hippocampus tissues. From each brain, the sample tissues were inserted into RNAlater™ (Ambion, Austin, TX) solution and were kept at −20°C.

### *Protein and total RNA isolation protocols*

The hippocampus tissues were transferred from RNAlater (−20°C) into cold TriReagent solution (Sigma, St. Louis, MO) and homogenized in a glass-Teflon homogenizer. The TriReagent suspensions were mixed thoroughly with chloroform and centrifuged (12,000 $g$, 15 min, 4°C). The organic phase (containing proteins) and the interphase (containing DNA) were further treated with 100% ethanol and centrifuged (10,000 $g$, 15 min, 4°C). The protein was precipitated with isopropanol and centrifuged (12,000 $g$, 15 min, 4°C). The protein pellet was washed several times with 0.3 $M$ guanidine hydrochloride in 95% ethanol, according to the manufacture's protocol (Sigma). Protein content was determined using the Bradford method. The aqueous phase containing the RNA was precipitated with isopropanol and centrifuged (12,000 $g$, 15 min, 4°C). The RNA pellet was washed twice with 70% ethyl alcohol (7500 $g$, 10 min), followed by one wash with 96% ethyl alcohol (12,000 $g$, 10 min), resuspended in diethylpyrocarbonate (DEPC)-treated water and incubated for 5 min at 56°C to facilitate resuspension. Total RNA aqueous solution was treated with DNase–RNase free (Roche Diagnostics, Mannheim, Germany) for 30 min at 37°C and subsequently was extracted by a round of phenol:chloroform:isoamylalcohol (25:24:1), followed by one round of chloroform:isoamylalcohol (24:1). After precipitation with sodium acetate (0.3 $M$) and isopropanol, the RNA pellet was washed as described above with 70% and 96% ethyl alcohol, resuspended in DEPC-treated water. RNA sample concentrations were determined by UV spectrophotometer at 260 nm. The quality of RNA was assessed by the absorbance at 260 nm/280 nm ratio and by agarose gel analysis through direct visualization of 18S and 28S rRNA bands.

### *Two-dimensional gel electrophoresis and image analysis*

Two-dimensional (2D) polyacrylamide gel electrophoresis was performed under conditions essentially as described by the manufacturer's protocol (Bio-Rad, Hercules, CA), using 110-mm pH 3–10 immobilized pH gradients (IPG) strips and Criterion (4–20% gels). Protein samples were dissolved in buffer containing 7 $M$ urea, 2 $M$ thiourea, 65 m$M$ dithiothreitol, 0.125% (vol/vol) Biolytes 3–10, 2% CHAPS, and bromophenol blue. For the first-dimension, 200 μg of protein were applied to a dehydrated IPG strip and isoelectric focusing was carried out at room temperature as follows: passive dehydration for 1 h and then at linear gradient from 50 V until

8000 V for 12 h. Subsequently, strips were placed on Criterion gels and the second-dimension separation was carried out at 200 V for 75 min. Following electrophoresis, gels were visualized by SeeBend Forte protein staining solution following the manufacturer's recommendation (Bio-Rad, CA). The gel images were acquired with a Bio-Rad Fluor-S Multi-Iimager and spots were indexed using the PDQuest 2-D software for comparisons and quantitation of 2D gel spots.

### *Gel proteolysis and mass spectrometry analysis*

Spot of proteins changed over or under 1.5-fold in abundance were further analyzed. The proteins in the gel were reduced with 10 m*M* DTT (60°C for 30 min) and modified with 100 m*M* iodoacetamide in 10 m*M* ammonium bicarbonate (room temperature for 30 min). The gel pieces were dehydrated with acetonitrile and rehydrated with 10% acetonitrile in 10 m*M* ammonium bicarbonate containing trypsin (Promega, CA) overnight at 37°C. The tryptic peptides were resolved by reverse-phase chromatography on 0.1 × 200-mm fused silica capillaries (J&W, CA, 100 micrometer ID) and packed with reversed phase material (Grace Vydac, CA). Subsequently, the peptides were eluted with linear 50 min gradients of 5–95% of acetonitrile with 0.1% formic acid in water at flow rates of 0.4 μl/min. Mass spectrometry (MS) was performed by an ion-trap mass spectrometer (LCQ-DecaXP, Finnegan, San Jose, CA) in a positive mode, using repetitively full MS scan followed by collision induces dissociation (CID) of the three most dominant ion selected from the first MS scan. The MS data were clustered and analyzed using the Pep-Miner (3) and Sequest software (University of Washington and Finnegan, San Jose) (82) searching the NR-NCBI database against numbers of database of human, mouse, rat, and bovine. A peptide was considered as high quality in Pep-Miner 80 and in the Sequest Xcore identification if the score was greater than 1.5 for singly charged peptides, 2.5 for doubly charged peptides, and 3 for triply charged peptides. The proteins were assigned to functional categories by approach used in PubMed database and the database for annotation, visualization, and integrated discovery 2.0 (DAVID 2.0) program.

### *Immunoblotting analysis*

For Western blot analyses, equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (NuPAGE 4–12% Bis-Tris electrophoresis gel (Invitrogen, Groningen, The Netherlands) and blotted onto polyvinylidene difluoride membranes (Millipore, MA). Membranes were treated with blocking buffer (5% dry milk in PBS or 5% dry milk, 0.05% Tween 20 in TBS). Primary antibodies were diluted in PBS or in TBS containing 5% BSA, 0.05% Tween 20, and incubated with membranes for 20 h at 4°C, followed by incubation (1 h at room temperature) in dilutions of horseradish peroxidase-conjugated secondary antibodies in the same buffer. Detection was achieved using Western blotting detection reagent, ECL (Amersham, Pharmacia, Little Chalfort, Buckinghamshire, UK). Quantification of results was accomplished by measuring the optical density of from the autoradiogram, using the computerized imaging program Bio-1D (Vilber Lourmat Biotech. Bioprof., Marne-La-Vallee Cedex, France). The following antibodies were used and the dilutions were performed according to the manufacture's protocol: rabbit polyclonal to ferritin heavy chain (GeneTex, Inc., TX) rabbit polyclonal anti-profilin 1 (Cell Signaling, Tech., MA) rabbit anti-peroxiredoxin 2 (Upstate, VA). The values of the labeled bands were normalized to monoclonal anti-β-actin (Sigma) intensity levels.

### *Atlas cDNA Microarray*

Hybridization array analysis was performed using Atlas Rat 1.2 cDNA expression arrays, including 1176 genes, according to the manufacturer's protocol (Clontech, Palo Alto, CA). Probes were generated from RNA pools and analyzed on cDNA arrays. Two independent experiments were performed. The membranes were exposed to phosphor screen (BAS MP-2040 image plate, Fuji Inc.,Tokyo, Japan) and the radioactive signals were detected with FLA-2000 scanner (Fuji Inc.). Quantification and analysis of the radioactive signals were done using AtlasImage™ 2.01 software (Clontech). The analysis was done by taking the log (base 2) of the ratio for the expressed genes and calculating their mean and standard deviation. Global normalization was based on the assumption that all of the genes in the array should have an average expression ratio equal to 1 and that the changes in expression of the individual genes balance out, so that the total quantity of RNA hybridized from each sample is the same (59). Changes with $\log_2$ (ratio) greater than two standard deviations of the mean were considered as significant (49). The genes were assigned to functional categories by approach used in PubMed database and the database for annotation, visualization, and integrated discovery 2.0 (DAVID 2.0) program.

### *Statistical analysis*

The data was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test. Differences between groups were considered significant if the difference between the groups reached a level of significance of $p < 0.05$.

## RESULTS

### *Proteomic analysis and immunoblotting validation*

To investigate the effect of aging on the overall expression of the proteins in the hippocampus, proteins were extracted from the hippocampal tissues of young and old rats, and analyzed in parallel by 2D gels. Analysis of specific spots revealed 200 proteins; among them, several proteins showed differential expression in the old rats compared with young rats and were identified by mass spectrometry and database searches. Table 1 lists the putative protein identifications and associated analysis information for each gel spot marked on the representative gel in Fig. 1. Mass spectrometry analysis allowed the identification of proteins related to neurodegenerative diseases, binding/chaperone family members, and metabolism/oxidation processes (Table 1).

Figure 2a presents the changes and the relative expression levels of three proteins, related to neurodegenerative diseases

TABLE 1. SUMMARY OF PROTEINS EXERTED DIFFERENTIAL EXPRESSION, IDENTIFIED BY MASS SPECTROMETRY IN HIPPOCAMPUS OF YOUNG AND OLD RATS

| *SSP* | *Gi-Accession #* | *Identified Protein* | *Peptide Patch Identified* | *% Coverage Matched Peptide* |
|---|---|---|---|---|
| **Neurodegenerative diseases** | | | | |
| 1701 | 13929098 | Neurofilament, light polypeptide | 19 | 44 |
| 5102 | 6978859 | Ferritin, heavy polypeptide 1 | 5 | 34 |
| 8504 | 62641274 | Tu translation elongation factor, mitochondrial protein | 13 | 36 |
| **Binding proteins/chaperons** | | | | |
| 1003 | 34881001 | Phosphoprotein enriched in astrocytes 15 PEA 15 | 3 | 33 |
| 4101 | 8393910 | Phosphatidylethanolamine binding protein | 9 | 66 |
| 7001 | 42476144 | Profilin 1 | 5 | 52 |
| 7502 | 54400730 | Chaperonin containing TCP1, subunit 2 (beta) | 9 | 32 |
| **Metabolism/oxidation** | | | | |
| 5307 | 16758446 | Isocitrate dehydrogenase 3 (NAD+) alpha | 7 | 24 |
| 2001 | 24233541 | Cytochrome c oxidase subunit Va | 4 | 38 |
| 4001 | 16758362 | Cytochrome c oxidase subunit Vb | 3 | 34 |
| 3102 | 34849738 | Peroxiredoxin 2 | 5 | 24 |

A peptide was considered as high quality in Pep-Miner 80 and in the Sequest Xcore identification if the score was greater than 1.5 for singly charged peptides, 2.5 for doubly charged peptides, and 3 for triply charged peptides. SSP, the identification number of the selected spot assigned by the image analysis software; Gi Accession #, Gi accession number of proteins; Peptide Match Identified, number of peptide match; % coverage match peptide, sequence coverage of matched peptide (%). For more detail explanation of the method, see text.

in the hippocampus of old rats, as compared with young rats. The neurofilament light polypeptide (NF-L), described previously to be associated with the development of amyotrophic lateral sclerosis (ALS) (35) and the ferritin heavy polypeptide 1, involved in iron regulation in brain of patients with PD (12) were both overexpressed in the hippocampus of old rats by ~8- and ~1.8-folds, respectively, versus young rats (Fig. 2b). The mitochondrial protein, the translation elongation factor Tu, associated with two mutations in mitochondrial encoded tRNA genes described in AD (23), was decreased in the hippocampus of the old by ~75%, compared with young rats.

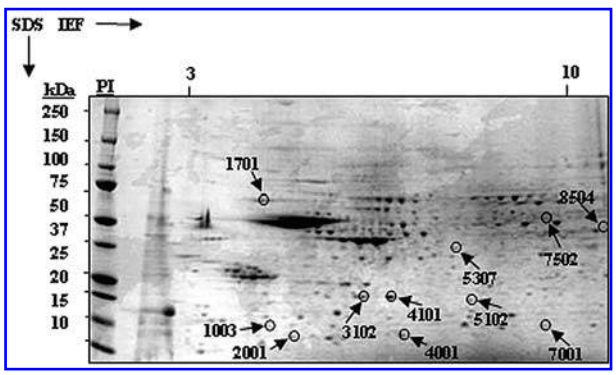


**FIG. 1. Representative 2D polyacrylamide gel electrophoresis of protein sample separated from hippocampal tissue of a young rat.** Each of the hippocampus samples from young ($n = 7$) and old ($n = 7$) rats was independently separated by 2D gel. The gels were visualized by SeeBend Forte protein staining solution. The spots numbers refer to identified proteins (see Table 1).

Figure 3 demonstrates the changes and relative expression levels of four proteins related to binding and chaperone families, measured in the hippocampus of old rats versus young rats. Phosphatidylethanolamine binding protein (PEBP) and phosphoprotein enriched in astrocytes 15 (PEA 15) were both decreased in the hippocampus of the old rats by ~10% and ~80%, respectively, compared with young rats (Fig. 3b). The chaperonin for actin containing t-complex polypeptide 1 (TCP1), subunit 2 (beta) was increased by about twofold and the actin-binding protein, profilin1 by about fourfold, in the hippocampus of old versus young rats (Fig. 3b).

Furthermore, the level of the catalytic subunit of the key regulatory enzyme in the Krebs cycle, isocitrate dehydrogenase 3 (NAD+) alpha, was increased by about twofold in the hippocampus of old versus young rats, as shown in Fig. 4. In contrast, the subunits Va and Vb of the mitochondrial enzyme cytochrome c oxidase and the antioxidant enzyme peroxiredoxin 2 were decreased by ~40%, ~15%, and ~20%, respectively, in the hippocampal tissues of aged rats compared with young rats (Fig. 4).

Moreover, to validate the proteomic data we have employed one-dimension immunoblotting assay of one important representative protein of each of the three clusters (Fig. 5). Indeed, Western blotting analysis confirmed the induction of ferritin and profilin1 levels, and reduction in peroxiredoxin 2 level in the hippocampus of old, compared with young rats. The difference between the extent of protein alterations determined by the proteomic analysis and Western blotting may be explained by the differences in the sensitivity thresholds of the methods (80).

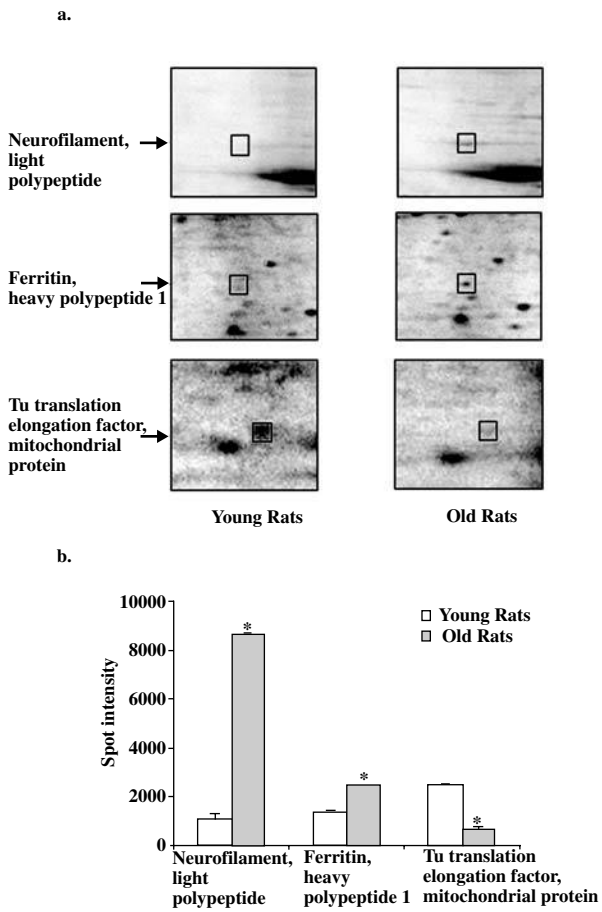


**FIG. 2. (a) Representative expended 2D images of proteins from hippocampus of young and old rats related to neurodegenerative diseases, clustered in Table 1: neurofilament light polypeptide, ferritin heavy polypeptide 1 and Tu translation elongation factor mitochondrial protein.** *Boxes* show the spot identified by mass spectrometry. (**b**) Quantification of 2D electrophoresis uses densitometry of matched spots. Data are expressed as mean ± SEM ($n = 7$); *$p < 0.05$ vs. young rats.

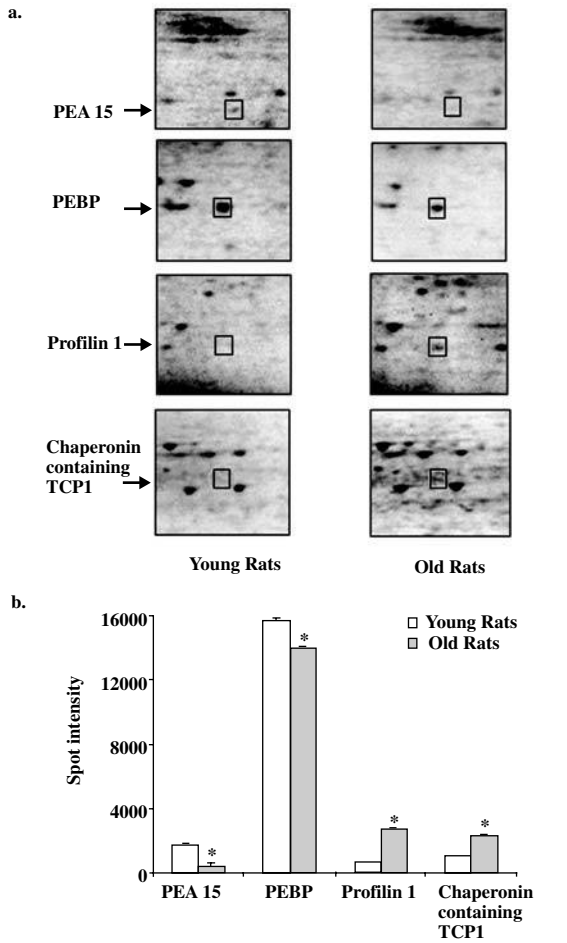


**FIG. 3. (a) Representative expended 2D images of proteins from hippocampus of young and old rats related to binding proteins and chaperon family members, clustered in Table 1: PEA 15, phosphoprotein enriched in astrocytes; PEBP, phosphatidylethanolamine binding protein; profilin 1 and chaperonin containing TCP1, subunit 2 (beta).** *Boxes* show the spot identified by mass spectrometry. (**b**) Quantification of 2D electrophoresis uses densitometry of matched spots. Data are expressed as mean ± SEM ($n = 7$); *$p < 0.05$ vs. young rats.

## *Gene expression profiling of hippocampal tissues from young and aged rats*

To gain insight into to the molecular alterations involved in aging and to generate complementary valuable information for the proteomic data, we performed gene expression profiling of the hippocampus of aged and young rats, using cDNA microarray analysis. Table 2 demonstrates various genes that were changed more than two standard deviations of the average ratio, selected and assigned to several clusters, including: neurodegenerative diseases, such as the familial AD-linked presenilin-1 (38); synapses process, such as the brain-specific isoform synaptotagmin IV; proliferation such as the retinoblastoma susceptibility-tumor suppressor gene, Rb; cell signaling pathways (*e.g.*, the guanine nucleotide release/exchange factor Ras-GRF (p140), PI3K, and 14–3-3 gamma). The majority of these genes were downregulated in the hippocampal tissues of old, compared with the levels in young animals. Similarly, genes related to metabolism, such as the iron homeostasis regulator gene, transferrin receptor (TfR), were diminished completely in the aged hippocampus samples, and those related to mitochondrial function and oxidation (*e.g.*, cytochrome *c* oxidase subunit Va) were decreased in the old rats, as described for the respective protein expression levels (Fig. 4).

## *Effect of rasagiline and ladostigil on gene expression profiling of hippocampal tissues from young and aged rats*

Profound understanding of the mechanisms that underlie various aspects of the aging process and age-related diseases (PD and AD) were examined by evaluating the effect of our potent neuroprotective compounds, the anti-Parkinson drug/MAO-B inhibitor, rasagiline, and the anti-Alzheimer drug, ladostigil. Thus, we assessed the effect of these drugs

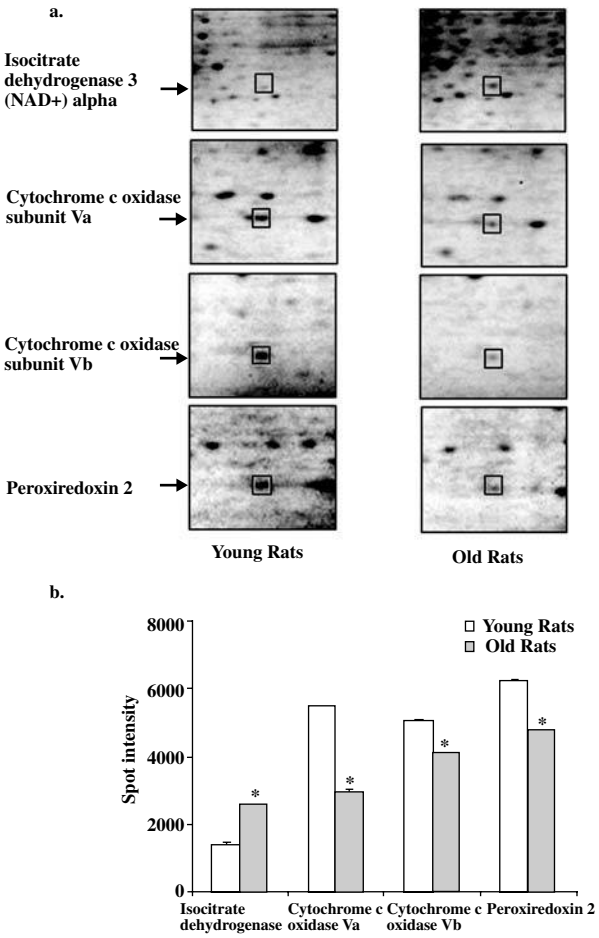


**FIG. 4. (a) Representative expended 2D images of proteins** from hippocampus of young and old rats related to metabolism and oxidation processes, clustered in Table 1: isocitrate dehydrogenase 3 (NAD+) alpha, cytochrome c oxidase subunit Va, cytochrome c oxidase subunit Vb, and peroxiredoxin 2. *Boxes* show the spot identified by mass spectrometry. (**b**) Quantification of 2D electrophoresis uses densitometry of matched spots. Data are expressed as mean ± SEM ($n = 7$); *$p < 0.05$ vs. young rats.

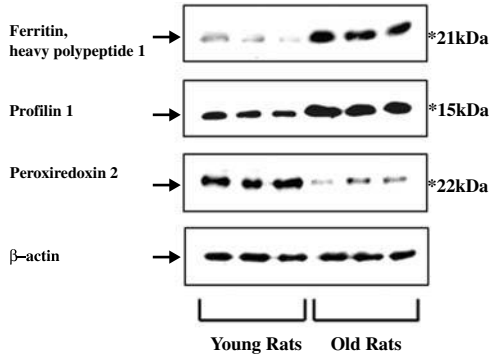


**FIG. 5. Representative Western blotting of one-dimension gel electrophoresis** detecting three important representative proteins from each of the clusters to validate the proteomic data obtained in 2D electrophoresis: ferritin heavy polypeptide 1, profilin 1, and peroxiredoxin 2. Three out of the seven hippocampal tissues from old and young rats are presented. The loading of the lanes was normalized to the levels of β-actin.

on functional genes in hippocampal tissues of young and old rats. Table 2 shows that rasagiline and ladostigil displayed similar effects on the expression of many genes. Especially in the old rats, rasagiline and ladostigil significantly downregulated the hippocampal levels of genes involved in: neurodegenerative diseases (*e.g.*, casein kinase I delta and the familial AD-linked presenilin-1); proliferation processes (*e.g.,* the tumor progression and metastasis genes, c-H-ras proto-oncogene transforming G-protein p21 and LIM domain kinase 2); and the electron transfer system, (NADPH-cytochrome P450 reductase, CPR). In addition, both drugs significantly reversed the effect of aging on several genes: upregulation of the brain-specific isoform synaptotagmin IV; the cell signaling gene, PI3K; the metabolic enzyme, aldolase C; the iron homeostasis regulator gene, TfR, and downregulation of 14-3-3 gamma and PKC gamma (γ) (Table 2).

## DISCUSSION

The aging process in the brain is not an outcome of one single gene or protein impairment, but rather a complex interaction of candidate genes and proteins that affect maintenance of the brain tissues (62). Currently, the human brain aging process is poorly understood, thus an important question is raised whether age-related neurodegenerative diseases, such as AD and PD, include components or by-products involved in aging. In this study, we focused on identification of proteomic and genomic elements involved in the brain aging process in an attempt to define common biomarkers for aging and neurodegenerative disease, and on this basis to investigate potential multifunctional neuroprotective therapeutic drugs for treatment of brain diseases (17, 84).

To date, there have been only a limited number of studies, particularly in aging, in which both DNA microarrays and proteomic technologies have been compared using the same tissue sample to describe potential parallel mechanism of genes and proteins regulation (53, 56, 58). However, discrepancies between mRNA expression and corresponding protein levels may be derived from post-translational modifications and post-transcriptional regulation. Nevertheless, evaluation of mRNA expression is regarded as a good indicator of the corresponding protein level and the advantage of a relatively easy screening tool may point to candidate families of genes and proteins. Indeed the current study has succeeded in generating a list of genes and proteins that are potentially involved in aging, including neurodegenerative diseases, metabolism, and oxidation processes.

### *Age-related alterations of genes and proteins in rat hippocampal tissues*

The protein levels of two isoforms of the energy metabolic enzyme, cytochrome *c* oxidase, subunits Va and Vb mitochondrial precursors, were decreased and the mRNA expression of subunit Va was downregulated as well in old

TABLE 2. GENE EXPRESSION ALTERATIONS IN YOUNG AND OLD RATS TREATED WITH/WITHOUT RASAGILINE AND LADOSTIGIL

| | | *8-month-old* | | | *27-month-old* | | |
|---|---|---|---|---|---|---|---|
| *Genebank* | *Gene* | *Control* | *Rasagiline* | *Ladostigil* | *Control* | *Rasagiline* | *ladostigil* |
| **Neurodegenerative diseases** | | | **(1 mg/kg, 30 days)** | | | **(1 mg/kg, 30 days)** | |
| L07578 | Casein kinase I delta; 49-kDa isoform | 9744 | 11886 | 3113 **D** | 9754 | 1552 **D** | 4844 **D** |
| D82363 | Presenilin 1 (PS1) | 16632 | 2138 **D** | 9150 | 7716 **d** | 2728 **D** | 10 **D** |
| AB0044 | Presenilin 2 (PS2) | 10486 | 9393 | 6240 | 7386 | 2183 **D** | 4663 |
| **Proliferation** | | | | | | | |
| D25233 | Rb; retinoblastoma susceptibility associated protein; tumor suppressor | 14876 | 12609 | 9644 | 2326 **d** | 5704 **U** | 8147 **U** |
| Y00047 | Proliferating cell nuclear antigen; cyclin | 16179 | 10755 | 9010 | 6660 **d** | 2135 **D** | 5515 |
| X17163 | c-Jun proto-oncogene; transcription factor AP-1 | 10969 | 10049 | 6759 | 7174 | 1287 **D** | 7497 |
| U35365 | Fyn proto-oncogene | 13202 | 12455 | 9139 | 9732 | 3991 **D** | 8393 |
| M13011 | c-H-ras proto-oncogene; transforming G-protein p21 | 13658 | 10 **D** | 9038 | 9894 | 2904 **D** | 1146 **D** |
| D31874 | LIM domain kinase 2 | 9080 | 10746 | 8763 | 9947 | 2966 **D** | 6357 **D** |
| **Synaptogenesis** | | | | | | | |
| L38247 | Synaptotagmin IV (SYT4) | 20540 | 17615 | 12977 | 6939 **d** | 8250 **U** | 11751 **U** |
| **Signal transduction** | | | | | | | |
| D30040 | RAC-alpha serine/threonine kinase (RAC-PK-alpha; AKT1) | 14107 | 11928 | 11153 | 8935 | 4841 **D** | 7828 |
| X67241 | Ras-GRF (p140); guanine nucleotide release/ exchange factor | 21733 | 8859 **D** | 6637 **D** | 706 **d** | 706 | 6259 **U** |
| D64045 | Phosphatidylinositol 3-kinase regulatory alpha subunit ( PI3K) | 11719 | 13206 | 9129 | 2228 **d** | 4922 **U** | 8689 **U** |
| X07287 | Protein kinase C gamma | 9488 | 8979 | 4403 **D** | 6114 | 1345 **D** | 7698 |
| AF003523 | Bcl-2-associated death promoter; Bad | 8943 | 6604 | 3412 **D** | 403 **d** | 1437 | 2089 |
| S55305 | 14–3-3 protein gamma subtype | 10 | 15400 | 13682 | 9421 **u** | 1262 **D** | 5546 **D** |
| **Metabolism/oxidation** | | | | | | | |
| X06984 | Aldolase C | 14639 | 12707 | 6946 **D** | 2460 **d** | 6113 **U** | 9011 **U** |
| M12919 | Fructose-bisphosphate aldolase A (ALDOA) | 12873 | 20276 **D** | 18042 | 17491 | 11639 | 17472 |
| J03753 | Brain calcium-transporting ATPase plasma membrane 1; calcium pump; PMCA1AB | 23502 | 21555 | 16104 | 7489 **d** | 9845 | 13110 |
| X15030 | Cytochrome c oxidase, subunit Va | 16782 | 18610 | 12493 | 9457 **d** | 6705 | 9981 |
| M12516 | NADPH-cytochrome P450 reductase (CPR) POR | 8335 | 7402 | 6100 | 5892 | 553 **D** | 2076 **D** |
| M58040 | Transferrin receptor protein; p90 | 3795 | 2501 | 2514 | 10 **d** | 1386 **U** | 1675 **U** |

Young ($n$ = 12) and old ($n$ = 12) rats were treated by gavage without (control)/with rasagiline or ladostigil (1 mg/kg, 30 days). Probes were generated from RNA pools and analyzed on cDNA arrays. Data are expressed as the level of quantified signals. Changes with $\log_2$ (ratio) greater than two standard deviations of the mean were considered as significant. **d**, significant signal of downregulation of gene expression and **u**, significant signal of upregulation of gene expression in hippocampus tissues from old compared with young rats; **D**, significant signal of downregulation of gene expression and **U**, significant signal of upregulation of gene expression in hippocampus tissues from drug-treated rats compared with age matched controls.

compared with young rats. Cytochrome *c* oxidase subunits Va and Vb, located at the matrix side of the multi-subunit complex of the mitochondrial respiratory chain enzyme cytochrome *c* oxidase, were shown to be involved in the transfer of electrons from cytochrome c to oxygen (32). Thus, decreased levels of these subunits in aging and age-related diseases, might affect the expression and activity of the other subunits and the entire complex of the cytochrome c oxidase. In support, previous immunohistochemical study demonstrated reduced levels of the nuclear coded subunits

V and the mitochondrial coded subunits II/III, as well as cytochrome *c* oxidase defects in the substantia nigra of old (70- to 90-year-old) subjects (26). Moreover, brains from AD patients showed 50–60% decreases in mRNA level of nuclear DNA-encoded subunit IV of cytochrome *c* oxidase (10).

Our study demonstrated reduced protein levels of the antioxidant enzyme, peroxiredoxin 2 (31), in hippocampus of old compared with young rats, in agreement with a previous study in aged human brains (11). Mitochondrial peroxiredoxins are involved in the redox regulation of cellular signaling and differentiation and play a role in the antioxidant defense system, which eliminate peroxides generated during cellular metabolism (60). Thus, it is likely that as a consequence of reduced activity of peroxiredoxin 2, which occurs during aging, the mitochondria will be subjected to enhanced OS. In addition, in the old rat brain, the levels of the mitochondrial key regulatory enzyme in the Krebs cycle, isocitrate dehydrogenase 3 (NAD+) alpha (79), were increased. These results are consistent with a previous report demonstrating high isocitrate dehydrogenase level in oxidative skeletal muscle fibers and in aging (37).

Proteomic studies had also identified alterations in another cluster of proteins, binding proteins and chaperons. The levels of profilin 1, an actin-binding protein (81), were increased in old rats, suggesting that the induction of this protein may reflect an outcome of cytoskeletal changes occurring in aging and PD brains (2). Additionally, TCP 1, a cytosolic molecular chaperone assisting in the proper folding of actin, tubulin and other cytosolic proteins (83), was increased in the hippocampus of old rats.

The abnormal accumulation of the major cytoskeletal constituent of neuronal cells, NF-L, in the hippocampus of old rats is in line with previous data demonstrating the NF-L intensification in the cytoplasm of neurons of sporadic ALS patients and other neurological diseases (40, 50). Previous studies described the involvement of NF-L oxidation in motor neuron degeneration, suggesting that OS is responsible for NF-L disassembly and aggregation leading to neuronal atrophy and death (18, 34). Additionally, the induction of the iron regulation protein, ferritin heavy polypeptide 1, in aged hippocampal tissues, is in accordance with previous reports describing irregular enlargement of ferritin heavy and light peptides in different neuronal and extraneuronal tissues of aged, PD, and AD human brains (12, 28, 73). Hence, the iron homeostasis regulator gene, TfR (13) was completely diminished in the aged versus young hippocampus. The association of neurodegenerative diseases and age-related diseases with OS, as well as iron, has been discussed previously and supported by extensive studies (27, 52, 87, 88). Increasing ROS in the mitochondria during aging has been suggested in causing DNA mutations and damaging the mitochondrial proteins (39). Taken together, alterations in proteins related to mitochondrial function and OS may reflect brain aging and underlie the higher risk for developing neurodegenerative diseases in older individuals (11). Further, we found in the hippocampus of old rats a reduction in the mitochondrial translation elongation factor Tu, associated with two mutations in mitochondrial encoded tRNA genes described in AD (23) and downregulation of PEBP, which was also shown to be reduced in the hippocampus of Tg2576 mice model of AD (19).

### *Effects of anti-Parkinson/anti-Alzheimer drugs on gene profiling in old rats*

The application of our results to neuropharmacology of neurodegenerative diseases (*e.g.*, PD and AD) was achieved by conducting pharmacogenomic studies of brains from young and old rats administered with two potent neuroprotective compounds, the anti-Parkinson drug/MAO-B inhibitor, rasagiline, and the anti-Alzheimer drug, ladostigil, (75, 77, 84). Rasagiline and ladostigil possess high homology in their effect on neuroprotective gene expression profile, in agreement with their similar mechanism of neuroprotective activity, demonstrated previously in cell culture and animal models of PD and AD (43, 44, 76, 77). Thus, the familial AD-linked genes, presenilin (PS) 1 and PS2, were downregulated by both rasagiline and ladostigil. This effect can be of value in reducing amyloid-β (Aβ) formation in senile plaques, since PS1 and PS2 are major components of the γ-secretase complex, which facilitates the generation of the Aβ peptide via intramembranous proteolysis of the amyloid precursor protein (APP) (33, 68, 72). Furthermore, rasagiline and ladostigil downregulated casein kinase 1 delta mRNA, associated with pathological hallmarks in several neurodegenerative diseases (67), and upregulated the gene linked to synapse formation, synaptotagmin IV, which is a brain-specific isoform of the synaptotagmin family, presumably involved in nervous system development (4) and neuroprotection processes (71). In fact, this effect reversed the reduction in synaptotagmin IV gene expression occurring in the hippocampus of old as compared to young rats. In this study, both drugs additionally regulated several genes involved in signal transduction pathways including the guanine nucleotide release/exchange factor Ras-GRF (p140), PI3K, 14–3-3 gamma protein, and PKCγ, indicating the essentiality of the activation of PKC–PI3K–AKT survival pathways in rasagiline and ladostigil mediated-neuroprotective effect. Our recent studies have clearly demonstrated that rasagiline given chronically 8 days post the Parkinsonian neurotoxin, methyl-4-phenyl-1,2,3,6-tetrahydropyridine, induced neurorescue/neuroprotection/neurogenesis of nigrostriatal dopamine neurons of mice and affected a number of cell signaling mediators associated with the tyrosine kinase receptor (Trk) pathway, including Ras, in parallel with a specific increase in the Trk-downstream effecter PI3K proteins (63). The present study, together with our previous one, may implicate a neuronal restoration of synaptic transmission by rasagiline and its anti-Alzheimer derivative drug, ladostigil, in aging and neurodegenerative diseases, suggesting a possible disease-modifying activity of these drugs. A larger clinical study is under way to confirm this (84, 86, 88).

The current study supports the concept that OS and mitochondrial dysfunction are important, and perhaps underlying, causes of the brain aging process, which remains the most important risk factor for neurodegenerative diseases. Further studies employing pharmacoproteomics will identify specific proteins involved in the neuroprotective mechanism of action

of rasagiline and ladostigil in old rat hippocampal tissues. The global profiling technologies of proteomics and genomics may help unraveling biomarkers, which will be of value for discovery of potential clinical markers and future novel therapeutic approach for age-related neurodegenerative diseases.

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## ABBREVIATIONS

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CID, collision induces dissociation; CPR, NADPH-cytochrome P450 reductase; 2D, two-dimensional; DEPC, diethylpyrocarbonate; GSH, reduced glutathione; HD, Huntington's disease; IPG, immobilized pH gradients; MAO, monoamine oxidase; MS, mass spectrometry; NF-L, light polypeptide; OS, oxidative stress; PD, Parkinson disease; PEA 15, phosphoprotein enriched in astrocytes 15; PEBP, phosphatidylethanolamine binding protein; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PS, presenilin; ROS, reactive oxygen species; SOD, superoxide dismutase; TCP, the chaperonin-containing t-complex polypeptide 1; TfR, transferrin receptor.

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