Neurobiology of Aging 58 (2017) 180–190

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Resilient protein co-expression network in male orbitofrontal cortex layer 2/3 during human aging

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ARTICLE INFO

Article history:
Received 8 February 2017
Received in revised form 23 June 2017
Accepted 24 June 2017
Available online 5 July 2017

Keywords:
Normal aging
Cortex
Layer 2/3
Proteomics and RNA-Seq

ABSTRACT

The orbitofrontal cortex (OFC) is vulnerable to normal and pathologic aging. Currently, layer resolution large-scale proteomic studies describing "normal" age-related alterations at OFC are not available. Here, we performed a large-scale exploratory high-throughput mass spectrometry–based protein analysis on OFC layer 2/3 from 15 "young" (15–43 years) and 18 "old" (62–88 years) human male subjects. We detected 4193 proteins and identified 127 differentially expressed (DE) proteins (p-value $\leq$0.05; effect size >20%), including 65 up- and 62 downregulated proteins (e.g., GFAP, CALB1). Using a previously described categorization of biological aging based on somatic tissues, that is, peripheral "hallmarks of aging," and considering overlap in protein function, we show the highest representation of altered cell-cell communication (54%), deregulated nutrient sensing (39%), and loss of proteostasis (35%) in the set of OFC layer 2/3 DE proteins. DE proteins also showed a significant association with several neurologic disorders; for example, Alzheimer's disease and schizophrenia. Notably, despite age-related changes in individual protein levels, protein co-expression modules were remarkably conserved across age groups, suggesting robust functional homeostasis. Collectively, these results provide biological insight into aging and associated homeostatic mechanisms that maintain normal brain function with advancing age.

1. Introduction

Aging is a major risk factor for the onset of brain disorders, and the cost for prevention and treatment of neurologic disorders in the aged population is a growing global burden (Silberberg et al., 2015). Existing treatment strategies are either minimal, nonspecific, or not available, mostly due to a limited understanding of the biology of normal brain aging.

Brain aging is a complex process that involves progressive and persistent changes occurring at the functional, neural network, morphologic, and molecular levels (Glorioso and Sibille, 2011; Yankner et al., 2008). At the functional level, there is an age-associated decline in cognition (Grady, 2012; Leal and Yassa, 2015; McQuail et al., 2015), motor function (Rosso et al., 2013), and mood (Fiske et al., 2009; Koenig and Blazer, 1992). At the network level, there are age-associated alterations in neuronal communication within and between various brain regions, specifically those subserving higher order cognitive functions; for example, prefrontal cortex (Andrews-Hanna et al., 2007; Geerligs et al., 2015). Finally, at the molecular level, changes in gene expression patterns have been reported for neurons and glia during human aging (Erraji-Benchekroun et al., 2005; Soreq et al., 2017; Yankner et al., 2008).

Recently, the molecular changes occurring during the course of aging in peripheral somatic tissues have been summarized and catalogued into 9 "hallmarks of aging" (Lopez-Otin et al., 2013). These include primary hallmarks, that is, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis; antagonistic hallmarks, that is, deregulated nutrient sensing,

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http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.023

mitochondrial dysfunction, and cellular senescence; and integrative hallmarks, that is, stem cell exhaustion and intercellular communication. The extent to which these peripheral hallmarks apply to brain tissue mainly comprised of nondividing cells has not been evaluated.

Much of the existing knowledge on human brain aging at the molecular level has been inferred from transcriptomic studies. A key benefit of this approach is the ability to obtain genome-wide information on cellular and molecular phenotypes. However, changes in gene expression may not systematically reflect at the protein level and are less correlative to biological functions. Proteomics, the study of large-scale protein expression, provides a different perspective on biological changes and can serve as a proxy for molecular and network/morphologic levels of aging. Compared with large-scale information obtained in a transcriptomic study (10–20,000 genes), mass spectrometry (MS)–based proteomic studies provide information on fewer proteins (5–10,000 proteins) due to inherent technical difficulties in protein analyses. These 2 approaches also differ in their dynamic regulation and sensitivity associated with methods and instrument detection (Schwanhausser et al., 2011; Zubarev, 2013). Importantly, both methods of analysis provide comprehensive and complementary information on the molecular and functional status of a cell or tissue.

The orbitofrontal cortex (OFC) is a part of the prefrontal cortex that is involved in cognitive tasks such as exteroceptive and interoceptive information processing, learning, and decision making related to emotion and reward stimuli (Kringelbach, 2005). These OFC-related behavioral modalities undergo age-dependent changes, such as a decline in delayed match and nonmatch to sample tasks and gray-matter volume loss (Lamar and Resnick, 2004; Resnick et al., 2007). This could result from intrinsic biological and morphologic changes (Dickstein et al., 2007; Resnick et al., 2007) in OFC, leading to altered communication between OFC and various other brain regions. Superficial cortical layer 2/3 cells have distinct roles in processing feedforward and feedback excitatory and inhibitory information. Age-dependent changes occurring in superficial layer 2/3 cells are hypothesized to play a significant role in major depression and other brain-related disorders (Northoff and Sibille, 2014; Sibille, 2013). Identifying changes occurring at the protein or mRNA level in layer 2/3 cells of OFC may provide information on how aging affects the critical functions of OFC. We have previously characterized age-dependent gene expression changes in combined gray matter samples from the OFC and dorsolateral prefrontal cortex (Erraji-Benchekroun et al., 2005), including upregulated transcripts mostly of glial origin and downregulated transcripts of neuronal origin largely related to neuronal communication and signaling.

Most MS-based proteomics studies performed until now on human aging cortical tissue have focused on a limited number of proteins (Chen et al., 2003; Manavalan et al., 2013; Pan et al., 2007; Xu et al., 2016a,b). Here, we performed an exploratory large-scale unbiased (i.e., no a priori selection) proteomic profiling of OFC (areas BA11/47) layer 2/3 in healthy aging subjects compared with a younger cohort. We tested the power of this approach to detect well-replicated gene changes in the brain and uncover novel age-related biological changes at the level of single proteins, biological pathways, and protein co-expression network modules. We further tested whether hallmarks of aging defined in peripheral somatic tissue apply to the mostly nondividing brain tissue. Based on the specificities of brain tissue and of the OFC layer 2/3 in particular, we predicted robust changes in protein expression related to neural communication, affecting the glutamate, gamma amino butyric acid, and glial systems.

2. Materials and methods

2.1. Human postmortem samples

Frozen postmortem samples from OFC (Brodmann areas 11 and 47) of 15 younger (<45 years) and 18 older (>60 years) healthy male subjects (Supplementary Table 1) were obtained from the Douglas-Bell Canada Brain Bank, Montreal, Canada (http://douglasbrainbank.ca/) using procedures approved by the Douglas Hospital Research Ethics Board. The Research Ethics Board also approved the study. The choice of age and sex of the cohort was based on (1) the availability of postmortem samples with reasonable matching of cofactors such as postmortem interval (PMI) and brain tissue pH; (2) although brain aging is a continuous process, age-related changes in the gene expression patterns are relatively homogeneous and negatively correlated in age groups <42 and >73 years (Lu et al., 2004); and (3) there are potential sex differences in brain aging (Coffey et al., 1998) and fewer female samples were available. Therefore, a male cohort was selected for this study. All subjects were free of psychiatric illness as evaluated from clinical files and, in some cases, standardized structured psychological autopsy of a family member. Neuropathologic examination of the brains did not show any signs of neurodegenerative disorders. None of the subjects had prolonged illness or suffering before death. Most subjects died from accident or cardiovascular events. Group means for PMI and brain pH were not statistically different. All tissue blocks were stored at −80 °C until further analysis.

2.2. Laser capture microdissection (LCM)

Tissue sections (20-μm thickness) from frozen brains were collected on polyethylene naphthalate membrane glass slides (Thermo Fisher Scientific, MA, USA) using Leica CM1950 cryostat (Wetzlar, Germany) and subsequently stained with thionin. Briefly, slides were fixed and washed in 75% and 50% ethanol for 5 minutes and 1 minute, and then stained with 0.2% thionin (Sigma-Aldrich, MI, USA) for 10 minutes and rinsed in Milli-Q water. The slides were then dehydrated in a graded ethanol series twice (in 50% ethanol, 75% ethanol, 95% ethanol, and 100% ethanol for 30 seconds each) and lastly with xylene for 3 minutes. Layer 2/3 of stained tissue was identified and captured using the ArcturusXT laser capture microdissection (LCM) system (Thermo Fisher Scientific, MA, USA) (Supplementary Fig. 1). A total of 30–40 mm^2 (~10–20 μg of total protein) of layer 2/3 was collected per sample based on previously described estimates (He et al., 2013; Wisniewski, 2013), out of which 15–20 mm^2 (approximately 250,000–300,000 cells) yielding ~5–10 μg of total protein. ~2.5 μg was used for MS analysis.

2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Collected layer 2/3 tissue samples were homogenized in lysis buffer containing, 50-mM HEPES, pH 8, 150-mM NaCl, 2% SDS (weight by volume), 1-mM EDTA, 1-mM dithiothreitol, 1× Protease Cocktail Inhibitor (Roche, Basel, Switzerland), and 1× PhosSTOP (Roche). The resulting mixture was vortexed briefly and subjected to 6 cycles of sonication for 1 minute each with intermittent 2-minute incubations on ice. The samples were then incubated at 99 °C for 5 minutes and spun down at 15,000 rpm for 20 minutes. The resulting supernatant (protein homogenate) was collected and stored at −80 °C. Total protein concentration was estimated using a silver stain with BSA standards (0.1–1.0 μg). 20 μg of protein per sample was then reduced using 10-mM dithiothreitol (Sigma-Aldrich) and 2-mM TCEP (Thermo Scientific, MA, USA) by

incubating at 60 °C for 15 minutes. The samples were then subjected to protein alkylation using 50-mM iodoacetamide (Sigma-Aldrich) in the dark for 30 minutes at room temperature. Subsequently, reduced and alkylated protein homogenates were added to prewashed/equilibrated Amicon Ultra-Millipore Protein 0.5-mL filters (Millipore, MA, USA) for filter-aided sample preparation as previously described in Scifo et al. (2015). The process of lysate preparation and liquid chromatography-tandem MS (LC-MS/MS) analysis from all subjects were performed in 4 batches with 15 pairs (young and old) and 3 separate samples for ensuring reproducibility between runs. We did not perform technical replicates based on the limited availability of the postmortem tissue but in pilot experiments, we determined that 1.5% SDS was optimum for protein extraction/downstream MS experiments and technical replicates yielded reproducible peptide identifications.

LC-MS/MS analysis was performed at the SPARC BioCentre (http://www.sickkids.ca/Research/SPARC/about-us/index.html) as previously described (Zhang et al., 2015). Briefly, a ultra performance liquid chromatography system (EASY-nLC 1000, Thermo Fisher Scientific, MA, USA) was used to load samples and peptides were separated on a 50-cm column. A nano-electrospray ion source (EASY-SPRAY, Thermo Fisher Scientific, MA, USA) was used to introduce peptides into an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific, MA, USA). Raw MS data was processed by MaxQuant (version 1.5.3.8) for label-free quantification (Cox and Mann, 2008) of the proteome. The following MaxQuant settings were used: fragment ion mass tolerance of 20 ppm, maximum 2 missed cleavages (Trypsin and Lys-C), fixed modification as carbamidomethylation of cysteine, and variable modification as oxidation of methionine and acetylation of protein N-terminal. False discovery rate was set at 1% at both peptide and protein levels in target/decoy. We filtered out contaminants and reverse sequences to minimize false positive results. Proteins with at least 2 peptides were accepted and used for further analysis. These settings resulted in the identification of 4193 proteins. Of these, 2321 proteins were observed in at least 70% of samples and were selected for further analysis (Supplementary Table 2). The "match between runs" feature in MaxQuant, which allows for the alignment of peptides across various samples based on retention time and m/z irrespective of MS runs was used. This feature may be exploited for testing the reproducibility of sample preparation and LC-MS/MS runs based on the percentage of identifications across different specimens.

Methods on LC-MS/MS data imputation and statistical analysis, functional analysis, weighted gene co-expression networks, RNA-Seq analysis, and immunoblotting are presented in the Supplementary Methods section.

3. Results

3.1. Human postmortem OFC layer 2/3 proteome during aging

To determine the effect of aging on OFC layer 2/3 proteome, we performed LC-MS/MS analysis on the LCM of layer 2/3 (Supplementary Fig. 1) from 15 "young" (15–43 years) and 18 "old" subjects (62–88 years; Supplementary Table 1) (see Supplementary Fig. 2 for experimental workflow). Our LC-MS/MS analysis followed by label-free quantification resulted in identifying 4193 proteins (Fig. 1A and Supplementary Table 2). Out of these proteins, 2321 proteins were observed in at least 70% of samples and further analyzed. Statistical analysis using a random intercept model (RIM) (Wang et al., 2012) for adjusting cofactors (pH and PMI) resulted in a total of 127 differentially expressed (DE) proteins (65 up- and 62 downregulated; Supplementary Table 3 and Fig. 1B). Several of the identified DE proteins have previously been shown to be affected by aging, for example, up- and downregulated proteins such as GFAP, APOD, and CALB1 (David et al., 1997; del Valle et al., 2003; Emmanuele et al., 2012). These results illustrate that the proteomic approach adopted in this study was able to identify the qualitative and quantitative changes at the OFC layer 2/3 proteome during normal aging.

3.2. Peripherally-derived hallmarks of aging are differentially represented in cortical layer 2/3 brain aging

We systematically investigated whether hallmarks of aging defined based on peripheral somatic tissue (Lopez-Otin et al., 2013) were represented by OFC layer 2/3 DE proteins. These proteins were manually categorized into the previously described 9 hallmarks of aging based on their functional annotations from GeneCards (Fishilevich et al., 2016). Results show that intercellular communication or altered neural communication (integrative hallmark) and deregulated nutrient sensing (antagonistic hallmark; Fig. 2) were the top 2 most significant biological processes implicated, representing 70 (54%) and 46 (39%) out of 127 DE proteins, respectively. Other represented primary, antagonistic, and integrative hallmarks of aging were loss of proteostasis (40 proteins; 35%) and cellular senescence (37 proteins; 31%). Other hallmarks were less represented, including stem cell exhaustion (16 proteins; 14%), mitochondrial dysfunction (16 proteins; 13%), and epigenetic alterations (8 proteins; 7%). Only 3 proteins (3%) were associated with the genomic instability hallmark and none were with the telomere attrition category. The fact that the cumulative percentage of DE representation exceeds 100% is due to the poly-functionality of many proteins. These results demonstrated an uneven representation of peripheral hallmarks of aging in OFC layer 2/3, with a high representation of altered neural communication as the top hallmark of aging and undetected evidence for telomere attrition, highlighting the specificity of brain tissue.

Consistent with the "Hallmark" analysis, we identified glutamate receptor signaling pathways as commonly enriched in our DE protein list using various unbiased functional analyses (i.e., GOrilla and ingenuity pathway analyses; Fig. 1C and Supplementary Table 4). Other enriched functional groups and canonical pathways include single-organism development and catabolic process, cell development, and cell signaling (Fig. 1C and Supplementary Tables 4.1 and 4.2). Although the results from our functional analysis support the "Hallmark" analysis, it did not provide detailed information on the subtype of biological processes altered during aging within hallmarks. Hence, we performed further gene ontology (GO)–based analysis on the set of DE proteins in the most represented hallmarks: altered neural communication, deregulated nutrient sensing, and loss of proteostasis. As seen in Supplementary Table 4.3, there was enrichment in GO annotations related to protein phosphorylation, synaptic compartment, and calcium transport processes in altered neural communication hallmark. In the deregulated nutrient sensing hallmark, as expected, there was enrichment in small molecule metabolic and catabolic processes. Interestingly, we observed enrichment in GO annotations related to lysosomal or endosomal, polypeptide formation and protein folding biological processes in loss of proteostasis hallmark. These findings suggest an overall imbalance in the synthesis and degradation of proteins during aging, which could eventually lead to altered synaptic communication within OFC layer 2/3. Further deregulation of these processes could lead to the development of neurologic disorders. Indeed, our functional analysis suggested for a link between the DE proteins (Fig. 1C) and several neurologic and neuropsychiatric disorders, for example, altered CALB1 levels have been implicated in normal aging and Alzheimer's disease (Lu et al.,

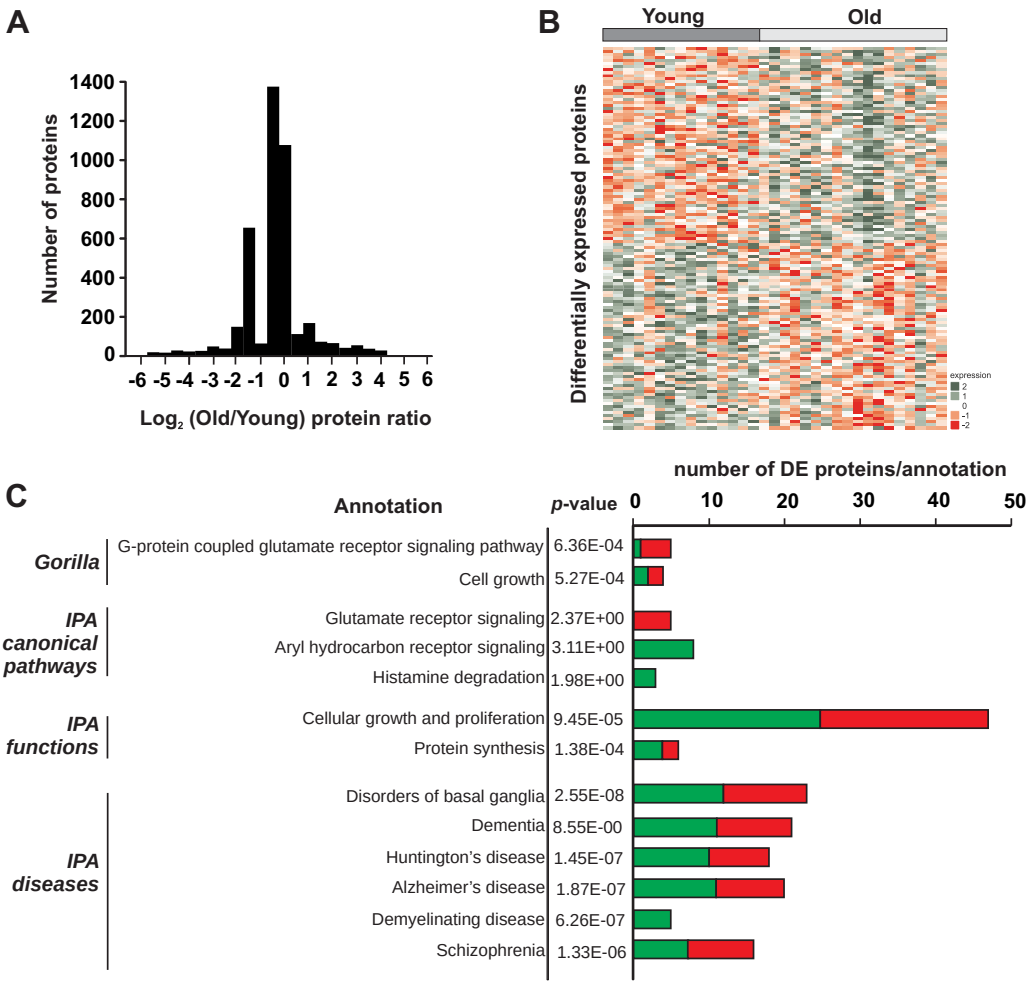

Fig. 1. Proteomic changes in OFC layer 2/3 during human aging. (A) The histogram distribution of $\log_2$ (old/young) protein ratios for 4193 proteins. (B) Heat map of differentially expressed (DE) proteins (127, 65 up- and 62 downregulated proteins, RIM coefficient effect size ± 0.263 with a p-value ≤ 0.05). Rows indicate proteins and columns indicate subjects. Color transition indicates upregulation (from red to green) and downregulation (from green to red) during aging. (C) Biological processes and disease categories are identified by functional analysis using GOrilla, g:Profiller, and IPA on DE proteins. Abbreviations: IPA, ingenuity pathway analysis; OFC, orbitofrontal cortex; RIM, random intercept model. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2014), schizophrenia (Fung et al., 2010), and depression (Maciag et al., 2010).

3.3. Co-expression network analysis on layer 2/3 proteome

Co-expression network analysis is useful in understanding complex alterations occurring during the aging process, where the resultant aging phenotype emerges from the convergence of numerous and incremental changes in deregulated expression of multiple proteins rather than from single deregulated protein expression (Gaiteri et al., 2014). To test this, we constructed co-expression protein networks using the WGCNA approach (Langfelder and Horvath, 2008).

3.3.1. Module assignment for old group proteins using young group proteins as reference

A network analysis in the young cohort identified 12 unique modules of co-expressed proteins. To determine the robustness of these modules to age-related changes, we performed a preservation analysis, first using module assignment in the young group as a reference. The results provided strong evidence for an overall preservation of modules between young and older subjects, including 6 modules with strong preservation ($Z_{summary} > 10$) and 6 modules with weak-to-moderate preservation ($Z_{summary}$ between 2 and 10) (Fig. 3A and C and Supplementary Tables 5.1 and 5.6). No modules showed values below 2, which is considered the threshold for complete loss of modularity between 2 conditions (Langfelder and Horvath, 2008).

Biological pathways represented in the conserved co-expression modules included multiple physiological processes, such as calcium homeostasis, synaptic transmission, neuron growth and development, DNA metabolism, protein metabolism and transport, consistent with the maintenance of basic cellular function with age (Table 1). To investigate age-related changes, we further analyzed the module with evidence of weakest preservation (magenta module; $Z_{summary} = 3.17$). The effect was driven by a loss of module density ($Z_{density} = 1.61$) rather than connectivity patterns ($Z_{connectivity} = 4.73$), suggesting highly resilient connectivity structure in the context of reduced connection strength (Fig. 3A and C and Supplementary Table 5.4). This module consisted of 64 proteins, with top enrichment for various functions related to altered protein homeostasis (i.e., proteostasis), including cotranslational protein targeting to membrane and protein localization to the endoplasmic reticulum. The module also included many ribosomal proteins such as RPL5, RPL18A, RPS3, and RPL19 involved in biological processes related to proteostasis. We also observed the

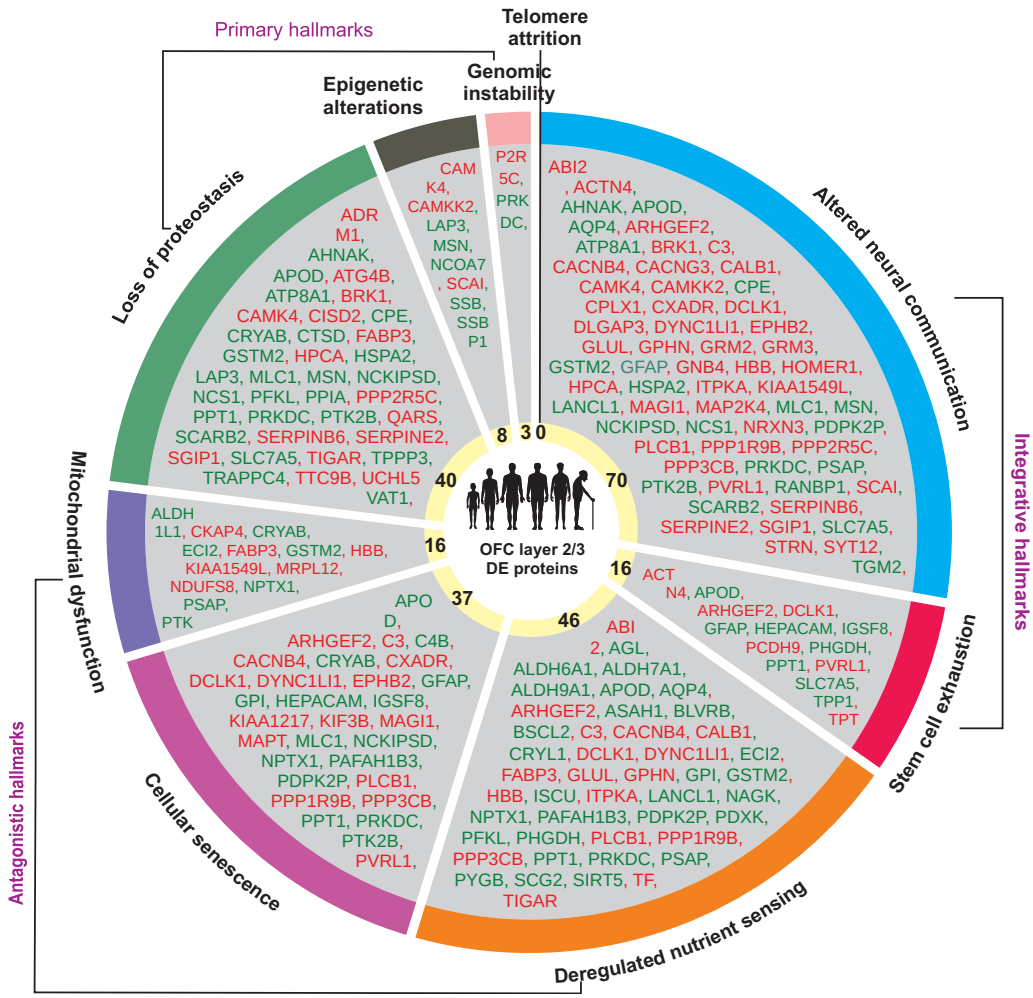

Fig. 2. Hallmarks of aging-based categorization of differentially expressed (DE) proteins. Classification of OFC layer 2/3 DE proteins into respective peripheral "hallmarks of aging" based on their function and involvement in the biological process using information from GeneCards. Altered neural communication (integrative) and deregulated nutrient sensing (antagonistic) hallmarks of aging are the 2 most represented in OFC layer 2/3 DE proteins. None of the DE proteins were associated with telomere attrition. Colored protein symbols indicate upregulation (green) and downregulation (red) during aging. Abbreviation: OFC, orbitofrontal cortex. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enrichment of GO biological process associated with mRNA transcription (Fig. 3E and Supplementary Table 5.4). Proteins showing highest intramodular connectivity (i.e., hub proteins) were MDH2, PEBP1, PPIA, and CTSB. Consistent with the functional enrichment in the overall module, these hub proteins are also associated with mitochondrial and proteostasis processes. Specifically, CTSB is involved in the proteolytic processing of amyloid precursor protein (Hook et al., 2008). The top 20% strongest connections among the 30 most connected proteins in this module are shown in Fig. 3E.

Together, these results suggest a remarkable overall conservation of functional modules between younger and older age, with a single module related to proteostasis showing moderate level of dysregulation. Of the 64 proteins in this least preserved co-expression module, 8 (e.g., BLVRB, PDXK) were found to be upregulated and none were downregulated in the older cohort compared with the young cohort in the original differential expression analysis (Fig. 3A and C and Supplementary Fig. 3).

3.3.2. Module assignment for young group proteins using old group proteins as reference

We next performed a network analysis restricted to the older cohort and identified 11 unique protein co-expression modules. Module preservation analysis using the old group as reference revealed a similar level of preservation than the opposite comparison, with all $Z_{summary}$ scores >4 (Fig. 3B and D and Supplementary Table 5.7). Biological pathways represented in the conserved co-expression modules included multiple basic processes associated with neuronal functions, such as nervous system development, cell-cell communication, membrane fusion/SNAP receptor activity, aspects of protein metabolism, RNA processing, and GTPase activity (Table 1). The least preserved module included 80 proteins (pink module; $Z_{summary} = 4.08$), with only 3 proteins upregulated and 1 downregulated identified in the differential expression analysis (Supplementary Fig. 3). This module showed enrichment for GO terms related to amino acid metabolism and catabolism, and to Ras activity (Supplementary Table 5.5). The top hub proteins in this module were ARHGAP26, DTNA, CNTNAP1, and EEF1D. Many of these proteins have enzymatic roles and aid in cellular organization and signaling processes. The top 20% strongest connections among the 30 most connected proteins in the pink module are shown in Fig. 3F. These results suggest that aging is associated with an increased function in protein network modules related to cellular organization and metabolism. Together, these results demonstrate that all protein co-expression modules observed in older subjects

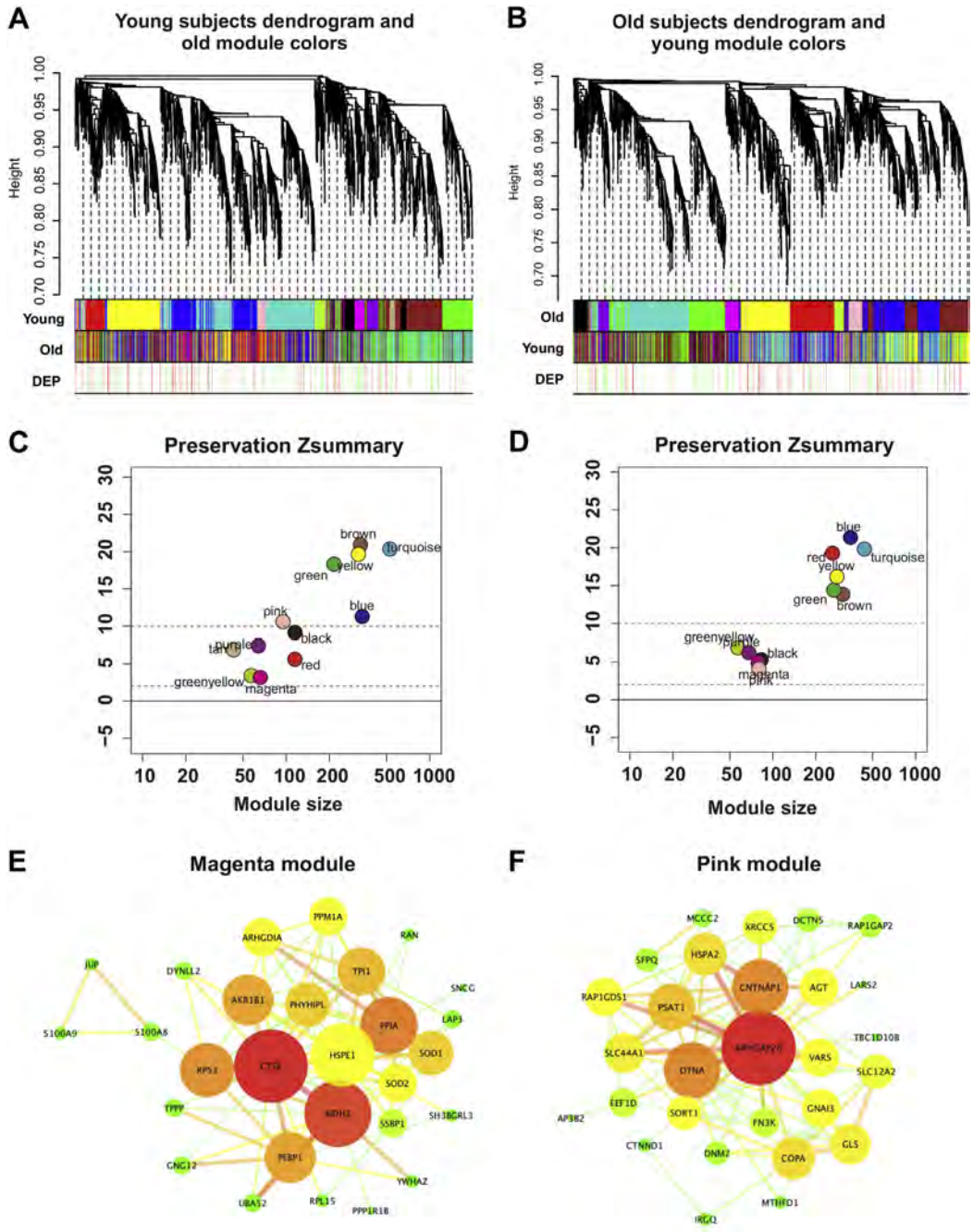

Fig. 3. Co-expression protein network module and preservation analysis on OFC layer 2/3 proteome. (A, B) Hierarchical clustering trees (dendrogram) of proteins based on young and old OFC layer 2/3 co-expression networks. The color rows beneath each dendrogram indicate module membership in young and old networks. The differentially expressed proteins (DEP) row demonstrates the distribution of either upregulated or downregulated proteins in the assigned color modules. (C, D) Identification of protein co-expression protein network modules in young and old subjects based on preservation $Z_{summary}$ statistics. (E, F) Modules magenta and pink are weak to moderately preserved in young and old subjects. The cytoscape diagrams demonstrate the top 20% of 30 most connected proteins. The node size and color represent the highest degree of connections (red; i.e., hubs) and the very few or lowest degree of connections (green). The distances between the nodes are arbitrary and have been modified in cytoscape for better visualization of the nodes and edges. Abbreviation: OFC, orbitofrontal cortex. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were already present in younger subjects, with only moderate evidence for changes in a single module with increased density in older subjects.

Overall, the combined analyses provide robust evidence for the presence of a conserved co-expression protein network during aging in layer 2/3 of the OFC, despite changes observed in the expression of proteins at the individual level.

3.4. RNA-Seq analysis on aging layer 2/3

Although changes occurring at OFC layer 2/3 proteome during normal aging can be identified using proteomic approaches, the sensitivity of the proteomic technique lags in comparison to assays of gene expression differences measured by the transcriptomics. Therefore, we performed an exploratory RNA-Seq analysis on layer

Table 1
GO term enrichment analysis on co-expression protein network modules. Protein lists associated with individual modules were input into g:Profiler for functional analysis. GO terms corresponding to biological processes were retrieved. Gray module which is not listed in the table represent "non-assigned" or background proteins (Langfelder and Horvath, 2008). The most enriched GO terms in each module are shown in the table

Preservation degree	Module	Reference group	Preservation $Z_{summary}$	Top GO terms
Weak to moderate	Magenta	Young	3.17	Proteostasis, mRNA transcription
	Greenyellow	Young	3.41	Nucleotide metabolism, synaptic compartment
	Red	Young	5.61	Organic molecule metabolism and carbohydrate catabolic process
	Tan	Young	6.87	Positive regulation of DNA metabolic process
	Purple	Young	7.40	Chemical synaptic transmission, cellular cation homeostasis
	Black	Young	9.15	Protein heterooligomerization, fibrinolysis
Strong	Pink	Young	10.58	Myeloid proliferation and differentiation, microtubule
	Blue	Young	11.27	Golgi vesicle transport, regulation of GTPase activity
	Green	Young	18.31	Nucleotide biosynthetic process, lipid catabolic process, cellular calcium ion homeostasis
	Yellow	Young	19.64	Mitochondrial membrane
	Turquoise	Young	20.35	Ribonucleotide binding
	Brown	Young	20.92	Neuron growth and development
Weak to moderate	Pink	Old	4.08	Amino acid metabolism and catabolism, Ras activity
	Magenta	Old	4.88	Zinc ion binding
	Black	Old	5.29	Organic molecule metabolism and carbohydrate catabolic process, gluconeogenesis
	Purple	Old	6.21	Cell-cell junction assembly
	Greenyellow	Old	6.85	Negative regulation of endocytosis, prostaglandin metabolic process
Strong	Brown	Old	13.84	Membrane fusion, SNAP receptor activity
	Green	Old	14.40	Nervous system development, active transmembrane transporter activity
	Yellow	Old	16.18	GTPase activity
	Red	Old	19.28	Regulation of GTPase activity
	Turquoise	Old	19.82	RNA processing
	Blue	Old	21.36	Cyclic nucleotide catabolic process, mRNA processing

Key: GO, gene ontology.

2/3 from a subset of young and old subjects, to assess correlation levels between proteomics and transcriptomics. We identified 55,106 transcripts of which 1633 were DE genes (Supplementary Table 6). Overall, gene transcript levels showed a moderate but highly significant correlation with protein levels (R ~0.27; p = 2.2E-27; Fig. 4A). Proteins showing moderate-to-high correlation (coefficients ranging from 0.38 to 0.92) with corresponding gene transcript levels included APOD, SLC7A5, MSN, PDXK, TGM2, NCKIPSD, CRYL1, HEPACAM, PTK2B, GSTM2, ALDH6A1, and AHNAK. We note that these correlations are present across age groups and their significance is thus likely robust to differences in protein co-expression modules between the young and the old groups.

Functional analysis using GOrilla, g:Profiler, and ingenuity pathway analysis for GO biological processes and Panther pathways on RNA-Seq DE genes demonstrated enrichment for synaptic transmission, proteostasis, Huntington disease, schizophrenia, cell

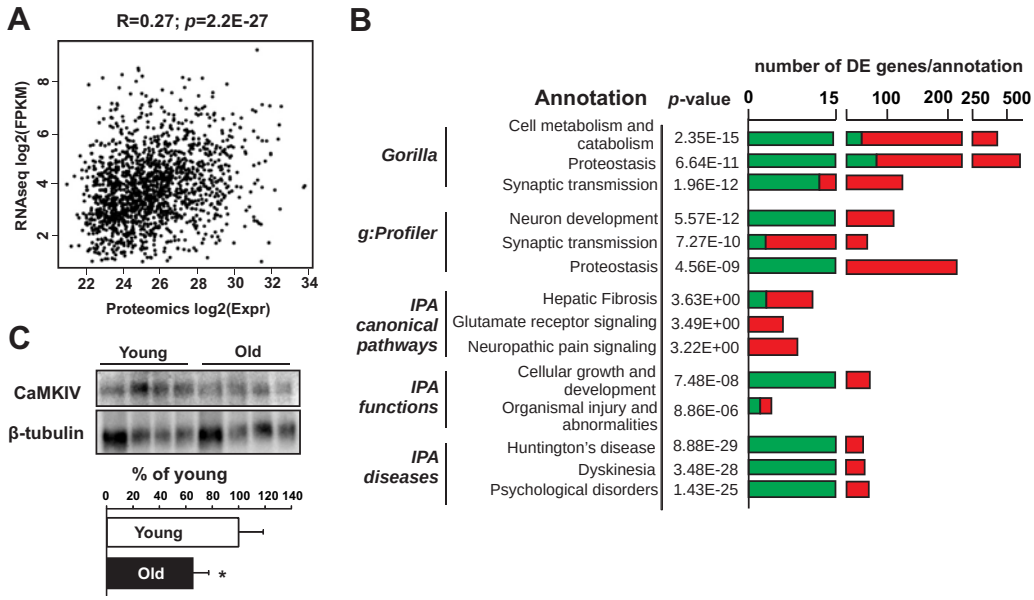

Fig. 4. RNA-Seq and Immunoblot validation of proteomic results. (A) A correlation plot between 1555 genes (RNA-Seq log2(FPKM)) and proteins log2(expression). A moderate correlation (R = 0.27) with highly significant p = 2.2e-27 is observed between genes and proteins at OFC layer 2/3. (B) Functional and network analysis using GOrilla, g:Profiler, and ingenuity pathway analysis (IPA) on differentially expressed (DE) genes. Several biological processes related to synaptic transmission, proteostasis, and cell development were commonly enriched in DE genes. Glutamate receptor signaling process was one of the significantly enriched pathways after IPA analysis on DE genes. IPA analysis also demonstrated a significant association between DE genes with several neurologic diseases including psychological disorders. (C) Immunoblot analysis for CAMKIV showed a significant reduction in protein levels in old subjects when compared with young subjects (* indicates $p < 0.05$, n = 3 different subjects, t-test). β-tubulin was used as loading control. Quantification of blots was performed using Image Lab software (Bio-Rad).

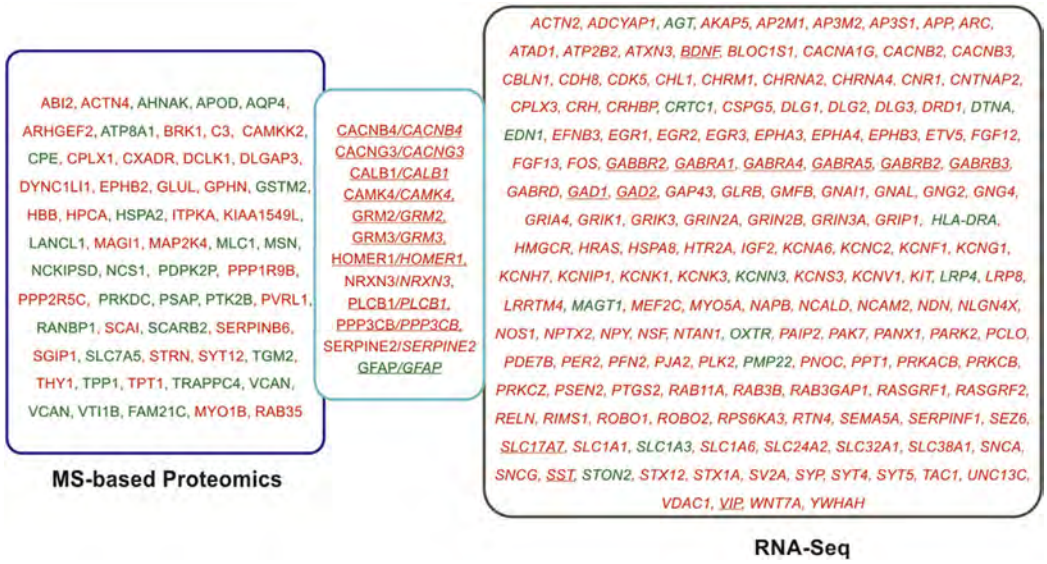

Fig. 5. Comparison of differentially expressed proteins and genes involved in altered neural communication/synaptic transmission hallmarks of aging. The left and right boxes show the list of differentially expressed proteins (70) and genes (173), respectively, categorized into altered neural communication. The middle box contains the proteins/genes that are present in both MS/MS proteomics and RNA-Seq–based approaches. Upregulated (green) or downregulated (red) protein or gene symbols are indicated in color. Synaptic genes or proteins replicated from our previous reports (Douillard-Guilloux et al., 2013; Erraji-Benchekroun et al., 2005) are underlined. Abbreviation: MS, mass spectrometry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metabolism, and catabolism among several others (Fig. 4B and Supplementary Tables 7.1–7.3), similar to the functional analysis performed on DE proteins (Fig. 1C). A direct comparison between DE genes and DE proteins involved in synaptic transmission suggested a more comprehensive view on the cell-cell or neural communication hallmark of aging. As seen in Fig. 5, a greater number of genes are detected at the RNA level (173) when compared with proteins (70) with only 11 in common. These results suggest that RNA-Seq analysis and proteomics approach yielded complementary results and can thus provide different perspectives on age-related biological changes.

3.5. Immunoblot validation

Lastly, we sought to validate our proteomic results by performing immunoblot on OFC layer 2/3 from a subset of randomly selected aging subjects. Consistent with proteomics results, the protein level of CAMKIV was reduced in old subjects (64.2% ± 12.2%) when compared with young (100% ± 18.8%, n = 3, $p = 0.031$, *t*-test) (Fig. 4C). We performed an immunoblot experiment for only one protein due to the small amount of tissue collected by LCM and the requirement for higher total protein concentration (~5–10 μg, with the total protein yield of layer 2/3 is ~5 μg) to run a conventional immunoblot.

4. Discussion

In this report, we present a proteomic snapshot of molecular changes occurring in male OFC layer 2/3 during "normal" human aging. Consistent with our prediction, we observed a robust age-associated alteration in protein levels related to neural communication, in line with previous findings at the RNA level (Douillard-Guilloux et al., 2013; Erraji-Benchekroun et al., 2005). We also observed a partial representation of peripheral "hallmarks of aging," consistent with the biological specificities of the brain, namely a mostly nondividing tissue. Finally, our protein co-expression analysis revealed a surprising high level of module conservation between younger and older subjects, suggesting a robust functional homeostasis in OFC layer 2/3 despite changes in single proteins.

It has been suggested that the OFC is selectively vulnerable to aging (Resnick et al., 2007) and age-dependent neuropsychiatric and neurodegenerative disorders (Frisoni et al., 2009; Lacerda et al., 2004; Tondelli et al., 2012), however, with mostly unclear mechanisms. Recent evidence suggests changes in the components of glutamate/gamma amino butyric acid system (i.e., excitation/inhibition balance) during aging within the OFC leading to intra- and inter-regional changes in neural communication (Legon et al., 2016). Altered excitation/inhibition balance observed in the OFC during normal aging could arise from intrinsic alterations occurring at the synaptic level. Indeed, several morphologic studies have attempted to provide a neuroanatomic basis for the age-related cognitive decline by demonstrating loss of synapses and regression of spines in human postmortem prefrontal cortex tissue (layers 2/3 and 5) (de Brabander et al., 1998; Morrison and Baxter, 2012). Although morphometric studies provide information on neuronal morphology, more functional information related to the vulnerability of synaptic transmission during aging is lacking. Our results support and provide indirect evidence regarding the age-associated loss at the synaptic level. Alterations in protein levels related to neural communication, although not always cohesive, were also observed in several targeted proteomic studies performed on human postmortem aging tissue (Chen et al., 2003; Manavalan et al., 2013; Pan et al., 2007; Xu et al., 2016a,b).

Our study, to the best of our knowledge, is the first to employ LCM combined with highly sensitive and powerful MS-based technology on a larger cohort of human postmortem tissue, specifically, layer 2/3 for investigating "normal" age-related changes. Based on earlier studies employing LCM combined with MS analysis on cancer tissue (He et al., 2013; Wisniewski, 2013), we reasoned that the starting material of 30–40 mm^2 tissue can provide anticipated protein identifications (4000–5000 proteins) that can be quantifiable and well within the dynamic range of a cell proteome (Zubarev, 2013). We identified 127 (5.5%) DE proteins out of 2321

proteins expressed in over 70% of our samples (out of 4193 total proteins) in our study after statistical analysis (Fig. 1A and B, Supplementary Tables 2 and 3). Our observation of 5.5% of DE proteins (including trademark aging proteins such as GFAP, CaMKIV, MAPT, and CALB1) during aging is in agreement with several MS-based aging studies (Chen et al., 2003; Manavalan et al., 2013; Pan et al., 2007; Xu et al., 2016a,b). However, numerous key differences should be noted in comparison to earlier studies such as: (1) investigation of age-related changes in a larger cohort of human subjects (33 vs. 4–16); (2) less starting material (30–40 mm^2 vs. whole cortex or mg of tissue); (3) region and layer specific resolution (OFC layer 2/3 vs. whole cortex and hippocampus); (4) male subjects compared with male and female or female subjects alone; and, finally, (5) cohesive results highlighting the changes in neural communication. It is important to note that our MS-based proteomic approach used in this study did not investigate post-translational modifications, for example, phosphorylation, acetylation on protein expression changes during normal aging, since analysis of post-translational modifications requires separate sample preparation, protocols, and analysis. Moreover, due to the limited availability of human postmortem samples, these studies were not replicated in an independent cohort. Nevertheless, the representation of 8 out of 9 hallmarks of aging, except telomere attrition (Fig. 2), in our DE proteins highlights the efficiency of the adopted proteomic approach in identifying age effects on the layer 2/3 proteome.

Our categorization of DE proteins into such hallmarks of aging (Lopez-Otin et al., 2013) has several advantages. It serves as a starting point for summarizing and understanding complex changes in biological processes during aging, provides information on the most age-affected biological processes at a given region of interest, and informs on which hallmark(s) of aging may remain unaffected (unless undetected) during brain aging. The brain is unique in its functions, anatomy, and physiology. Due to its high reductive environment and disproportionate requirements in energy, it is particularly vulnerable to the deleterious effects (specifically, loss of homeostatic balance in proteostasis and mitochondrial dysfunction) of aging (Patel, 2016; Sibille, 2013). Our observation of alterations in biological processes related to altered neural communication, cellular senescence (integrative hallmarks or culprits of the phenotype), mitochondrial dysfunction, deregulated nutrient sensing (antagonistic hallmarks) and loss of proteostasis (primary hallmarks) is consistent with a brain-specific aging program (Lopez-Otin et al., 2013; Patel, 2016; Sibille, 2013; Fig. 2). It is possible that our categorization of a limited number of DE proteins (118) can bias toward one or few particular hallmarks of aging. However, identification of enrichment in synaptic transmission and proteostasis-related GO terms on DE proteins (127) or a large number of RNA-Seq DE genes (1633) using unbiased functional analysis (Figs. 1 and 4) validates our categorization process. Although we observed a moderate, yet highly significant correlation between protein and gene expressions (Fig. 4A) in our data set, a closer examination of the molecular correlates of synaptic transmission, genes, and proteins (Fig. 5), provided comprehensive information on the type of altered molecules (increased or reduced) during aging. Many of these synaptic gene expression changes in layer 2/3 were also previously observed in our earlier and other reports (e.g., *HOMER1, CAMKV, GFAP, CALB1 and GABRA5*) (Douillard-Guilloux et al., 2013; Erraji-Benchekroun et al., 2005; Lu et al., 2004; Soreq et al., 2017) suggesting that they may constitute potential markers of brain aging. Interestingly, functional analysis on DE genes/proteins of the human OFC layer 2/3 revealed a significant association with several brain disorders (Figs. 1C and 4B), suggesting that any further alterations induced by genetic or environmental factors could lead to the development of age-dependent brain disorders. Our interpretation is further supported by a recent animal model study demonstrating the importance of medial prefrontal cortex layer 2/3 in stress-induced depressive behaviors (Shrestha et al., 2015).

An intriguing question in aging neuroscience concerns the mechanism underlying selective vulnerability of brain regions (e.g., OFC) and eventual decline in physiological functions. Results from this study suggest a primary role for deregulated processes in "primary" hallmarks of aging, such as loss of proteostasis, potentially activating changes in "antagonistic" hallmarks of aging, such as deregulated nutrient sensing, and consequently affecting the "integrative" hallmark, such as altered neural communication (Lopez-Otin et al., 2013). Our proteomic results are consistent with this putative sequence of events occurring in OFC layer 2/3 during normal aging.

Co-expression protein or gene networks can provide a framework for understanding complex intrinsic age-altered interactions between genes and proteins and how these can influence the overall communication within OFC layer 2/3. Surprisingly, we found that the majority of protein co-expression module structure was preserved between the old and the young groups (Fig. 3) including neuron growth/development and nucleotide metabolic/catabolic process (Table 1 and Supplementary Tables 5.6 and 5.7). Conserved protein co-expression suggests conserved integrated functions across those proteins, which here suggests remarkable, and somewhat non-intuitive, functional conservation between the young and old brain. The only module that appeared to lose its cohesion going from young to old was enriched for proteins involved in one of the "hallmarks of aging," namely proteostasis including transcriptional and translational regulations of mRNA. This finding suggests that, in addition to changes in individual protein levels, there may be a selective age-related weakening of cohesion within protein-protein interaction networks involved in these complex functions. Especially, the loss of module cohesion was driven by reduced strength of connections, rather than specific protein-to-protein connectivity, which was largely preserved. Interestingly, while none of the individual proteins in each of these modules were found to be downregulated in the old group, several of them were upregulated. Thus, it is possible that the increase in individual protein levels may be a compensatory response to offset a loss of cohesion in a larger functionally complex protein network, to maintain functional homeostasis. The module preservation was stronger when using the old group as a reference, suggesting that fewer new modules or module properties are gained with aging. Indeed, evidence for gain of modules with age consisted of modest changes in one module enriched for pathways related to amino acid metabolic processes and cytoskeleton organization, indicating that healthy aging may be associated with slightly stronger cohesion in complex protein networks involved in these functions.

Despite the limited number of identified DE proteins (proteomics) in comparison with DE genes (transcriptomics), the high level of convergence between both techniques at the molecular level indicates that they are complementary in gaining biological insights on age-related changes in a given region of interest. The combined RNA-Seq and proteomic results on OFC layer 2/3 further suggests changes in biological processes such as protein synthesis, folding, and degradation (loss of proteostasis) which could potentially initiate deregulated nutrient sensing and consequently alter protein phosphorylation and calcium transport at synaptic compartments (Figs. 1C and 4B, Supplementary Tables 4 and 7). Overall, altered proteostasis, including imbalance in protein synthesis, folding (e.g., altered heat shock proteins), and degradation, may represent a key initiating mechanism underlying "normal" aging changes at OFC. This is particularly important in light of an emerging concept that altered proteostasis could lead to age-related neurodegenerative disorders (Dattilo et al., 2015).

In summary, our proteomic analysis of human male OFC layer 2/3 results suggests a surprising degree of functional homeostasis, as indicated by the preservation in protein co-expression networks despite significant alterations at individual protein levels associated with primary, antagonistic, and integrated "hallmarks" of aging. This suggests homeostatic mechanisms at the network level that are resilient to age-related perturbations in individual proteins. It remains to be seen whether changes in protein co-expression networks occur in layer 2/3 in the context of brain disorders. In future studies, similar MS-based analysis may be used to determine whether these results are consistent across cortical layers, or if they mostly reflect age-related changes occuring in neural networks engaged in cortico-cortical communication.

Disclosure statement

The authors have no actual or potential conflicts of interest.

Acknowledgements

We thank Drs. Paul Taylor and Jonathan Krieger, SPARC Center for LC-MS/MS analysis. This work was supported by the National Institute of Mental Health (NIMH) R01 MH093723-05 and the Campbell Family Mental Health Research Institute Operating Award to Etienne Sibille.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2017.06.023.

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