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

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Abstract

The orbitofrontal cortex (OFC) is vulnerable to normal and pathological 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 4,193 proteins and identified 127 differently expressed proteins (DE) (p -value < 0.05 ; effect size $> 20\%$), including 65 up- and 62 down-regulated proteins (e.g., GFAP, CALB1). Using a previously-described categorization of biological aging based on somatic tissues, i.e., peripheral “hallmarks of aging”, and considering overlap in protein function, we show

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Conflict of Interest. None

SUPPLEMENTAL INFORMATION

Supplementary information is available at the *Neurobiology of Aging* journal’s website (<http://www.neurobiologyofaging.org>).

Disclosure

All the authors declare no conflicts of interest relevant to the study and transfer copyright of the manuscript to Neurobiology of Aging.

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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 neurological disorders, e.g., 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.

Keywords

Normal aging; cortex; layer 2/3; proteomics and RNA-Seq

INTRODUCTION

Aging is a major risk factor for the onset of brain disorders, and the cost for prevention and treatment of neurological disorders in the aged population is a growing global burden (Silberberg et al., 2015). Existing treatment strategies are either minimal, non-specific 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, morphological 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, e.g., 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 nine "hallmarks of aging" (Lopez-Otin et al., 2013). These include primary hallmarks, i.e., genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis; antagonistic hallmarks, i.e., deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence; and integrative hallmarks, i.e. stem cell exhaustion and intercellular communication. The extent to which these peripheral hallmarks apply to brain tissue comprised mainly of non-dividing 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/morphological levels of aging. Compared to 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 two approaches also differ in their dynamic regulation and sensitivity associated with methods and instrument detection (Schwanhauser 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 non-match to sample tasks and gray-matter volume loss (Lamar and Resnick, 2004; Resnick et al., 2007). This could result from intrinsic biological and morphological changes (Dickstein et al., 2007; Resnick et al., 2007) in OFC, leading to altered communication between OFC and various other brain regions. Superficial cortical layers 2/3 cells have distinct roles in processing feedforward and feedback excitatory and inhibitory information. Age-dependent changes occurring in superficial layers 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 critical functions of OFC. We have previously characterized age-dependent gene expression changes in combined gray matter samples from the OFC and dorsolateral PFC (Erraji-Benchekroun et al., 2005), including up-regulated 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; Xu et al., 2016b). 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 to 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 non-dividing 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, GABA, and glial systems.

MATERIALS AND METHODS

Human postmortem samples

Frozen postmortem samples from OFC (Brodmann areas 11 and 47) from 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 a) the availability of postmortem samples with reasonable matching of cofactors such as postmortem interval (PMI) and brain tissue pH; b) 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); c) 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. Neuropathological 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.

Laser Capture Microdissection (LCM)

Tissue sections (20 μm thickness) from frozen brains were collected on PEN 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 min and 1 min, and then stained with 0.2% Thionin (Sigma-Aldrich, MI, USA) for 10 min 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 sec each) and lastly with Xylene for 3 min. Layer 2/3 of stained tissue was identified and captured using the ArcturusXTTM 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.

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

Collected layer 2/3 tissue samples were homogenized in lysis buffer containing, 50mM HEPES, pH 8, 150mM NaCl, 2% SDS (weight by volume), 1mM EDTA, 1mM DTT, 1X Protease Cocktail Inhibitor (Roche, Basel, Switzerland) and 1X PhosSTOP (Roche). The resulting mixture was vortexed briefly and sonicated six times for 1 minute each with 2-minute rest in between. The samples were then incubated at 99°C for 5 minutes and spun down at 15,000 rpm for 20 min. 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 (DTT) (Sigma-Aldrich) by incubating at 60°C for 15 min. The samples were then subjected to protein alkylation using 50mM Iodoacetamide (IAA) (Sigma-Aldrich) in the dark for 30 min 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 (FASP) as previously described in (Scifo et al., 2015). The process of lysate preparation and LC-MS/MS analysis from all subjects were performed in four batches with 15 pairs (young and old) and three separate samples for ensuring reproducibility between runs. We did not performed biological or technical replicates based on: a) the limited availability of the postmortem tissue and b)

pilot experiments using different detergent concentrations for protein extraction (3 technical replicates at 3h MS runs) yielded reproducible and higher number of 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 UPLC system (Esay-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 two missed cleavages (Trypsin and Lys-C), fixed modification as carbamidomethylation of cysteine, variable modification as oxidation of methionine and acetylation of protein N-terminal. False discovery rate (FDR) 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 two peptides were accepted and used for further analysis. These settings resulted in the identification of 4,193 proteins. Out of these, 2,321 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 utilized. 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.

RESULTS

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 LCM-captured 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 4,193 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 proteins (65 up- and 62 down-regulated) (Supplementary Table 3 and Fig. 1B). Several of the identified differentially expressed proteins have previously been shown to be affected by aging, for example, up- and down-regulated 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 qualitative and quantitative changes at the OFC layer 2/3 proteome during normal aging.

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 differentially-expressed proteins. These proteins were manually categorized into the previously described nine 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 two top most significant biological processes implicated, representing 70 (54%) and 46 (39%) out of 127 differentially expressed 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 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 differentially expressed protein list using various unbiased functional analyses (i.e., Gorilla and IPA analysis; 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 Table 4.1 and 4.2). Although the results from our functional analysis support the “Hallmark” analysis, it did not provide detailed information on the sub-type of biological processes altered during aging within hallmarks. Hence, we performed further gene ontology (GO)-based analysis on the set of differentially expressed 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 neurological disorders. Indeed, our functional analysis suggested for a link between the differentially expressed proteins (Fig. 1C) and several neurological and neuropsychiatric disorders, for example, altered CALB1 levels have been implicated in normal aging and Alzheimer’s disease (Lu et al., 2014), schizophrenia (Fung et al., 2010), and depression (Maciag et al., 2010).

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).

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 ($Z_{\text{summary}} > 10$) and 6 modules with weak to moderate preservation (Z_{summary} between 2 and 10) (Fig. 3A, C and Supplementary Table 5.1 and 5.6). No modules showed values below 2, which is considered the threshold for complete loss of modularity between two 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 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; $Z_{\text{summary}} = 3.17$). The effect was driven by a loss of module density ($Z_{\text{density}} = 1.61$) rather than connectivity patterns ($Z_{\text{connectivity}} = 4.73$), suggesting highly resilient connectivity structure in the context of reduced connection strength (Fig. 3A, 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 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 proteolytic processing of amyloid precursor protein (APP) (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. Out of the 64 proteins in this least preserved co-expression module, 8 (e.g., BLVRB, PDXK) were found to be upregulated, and none downregulated in

the older cohort compared to the young cohort in the original differential expression analysis (Fig. 3A, C and Supplementary Fig. 3).

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, 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; $Z_{\text{summary}} = 4.08$, 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 role 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 coexpression modules observed in older subjects 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.

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 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 1,633 were differentially expressed genes (Supplementary Table 6). Overall, gene transcript levels showed a moderate but highly significant correlation with protein levels ($R \sim 0.27$; $p = 2.2E-27$) (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 group.

Functional analysis using Gorilla, g:profiler and IPA for GO biological processes and Panther pathways on RNA-Seq differentially expressed genes demonstrated enrichment for synaptic transmission, proteostasis, Huntington disease, schizophrenia, cell metabolism and catabolism among several others (Fig. 4B and Supplementary Table 7.1–7.3), similar to the functional analysis performed on differentially expressed proteins (Fig. 1C). A direct comparison between differentially expressed genes and differentially expressed 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.

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 ($64.2 \pm 12.2\%$) when compared with young ($100 \pm 18.8\%$, $n=3$, $p=0.031$, t-test) (Fig. 4C). We performed immunoblot experiment only for one protein due to the small amount of tissue collected by laser capture microdissection and due to the requirement of higher total protein concentration ($\sim 5\text{--}10\text{ }\mu\text{g}$, with the total protein yield of layer 2/3 is $\sim 5\text{ }\mu\text{g}$) to run a conventional immunoblot.

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 non-dividing tissue. Finally our protein co-expression analysis revealed a surprising high level of module conservation between young and older subjects, suggesting a robust functional homeostasis in OFC layer 2/3 despite changes in single proteins.

It has been suggested that 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/GABA system (i.e., excitation/inhibition balance) during aging within the OFC leading to intra- and inter-regional changes in neural communication (Lego et al., 2015). Altered excitation/inhibition balance observed in the OFC during normal aging could arise from intrinsic alterations occurring at the synaptic level. Indeed, several morphological studies have attempted to provide a neuroanatomical 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; Xu et al., 2016b).

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 starting material of 30–40 mm² tissue can provide anticipated protein identifications (4,000–5,000 proteins) that can be quantifiable and well within the dynamic range of a cell proteome (Zubarev, 2013). We identified 127 (5.5%) differentially expressed out of 2,321 proteins expressed in over 70% of our samples (out of 4,193 total proteins) in our study after statistical analysis (Fig. 1A and B, supplementary tables 2 and 3). Our observation of 5.5% of differentially expressed proteins (including trademark aging proteins such as GFAP, CaMKIV, MAPT, 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; Xu et al., 2016b). However, numerous key differences should be noted in comparison to earlier studies such as: a) investigation of age-related changes in larger cohort of human subjects (33 vs. 4–16; b) less starting material (30–40 mm² vs. whole cortex or mg of tissue); c) region and layer specific resolution (OFC layer 2/3 vs. whole cortex and hippocampus); d) male subjects compared to male and female or female subjects alone; and, finally, e) cohesive results highlighting changes in neural communication. It is important to note that our MS-based proteomic approach used in this study did not investigate posttranslational modifications, e.g., phosphorylation, acetylation on protein expression changes during normal aging, since analysis of posttranslational modifications requires separate sample preparation, protocols, and analysis. Moreover, due to limited availability of human postmortem samples, these studies were not replicated in independent cohort. Nevertheless, representation of eight out of nine hallmarks of aging, except telomere attrition (Fig. 2), in our differentially expressed proteins highlights the efficiency of the adopted proteomic approach in identifying age effect on layer 2/3 proteome.

Our categorization of differentially expressed 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 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 differentially expressed proteins (118) can bias towards one or few particular hallmarks of aging. However, identification of enrichment in synaptic transmission and proteostasis related GO terms on differentially expressed proteins (127) or a large number of RNA-Seq differentially expressed genes (1633) using unbiased functional analysis (Figs. 1 and 4) validates our categorization process. Although we observed a moderate yet highly significant correlation between proteins and genes expression (Fig. 4A) in our data set, a closer examination of 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, 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 differentially expressed 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 (for example, 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 group (Fig. 3) including neuron growth/development, nucleotide metabolic/catabolic process (Table 1 and Supplementary Table 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 regulation 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, in order 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 differentially expressed proteins (proteomics) in comparison to differentially expressed 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, 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 occurring in neural networks engaged in cortico-cortical communication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Andrews-Hanna JR, et al. Disruption of large-scale brain systems in advanced aging. *Neuron*. 2007; 56(5):924–935. [PubMed: 18054866]
- Chen W, et al. Proteomic comparison between human young and old brains by two-dimensional gel electrophoresis and identification of proteins. *Int J Dev Neurosci*. 2003; 21(4):209–216. [PubMed: 12781788]
- Coffey CE, et al. Sex differences in brain aging: a quantitative magnetic resonance imaging study. *Arch Neurol*. 1998; 55(2):169–179. [PubMed: 9482358]
- Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol*. 2008; 26(12):1367–1372. [PubMed: 19029910]
- Dattilo S, et al. Heat shock proteins and hormesis in the diagnosis and treatment of neurodegenerative diseases. *Immun Ageing*. 2015; 12:20. [PubMed: 26543490]
- David JP, et al. Glial reaction in the hippocampal formation is highly correlated with aging in human brain. *Neuroscience letters*. 1997; 235(1–2):53–56. [PubMed: 9389594]
- de Brabander JM, et al. Layer-specific dendritic regression of pyramidal cells with ageing in the human prefrontal cortex. *Eur J Neurosci*. 1998; 10(4):1261–1269. [PubMed: 9749780]
- del Valle E, et al. Apolipoprotein D expression in human brain reactive astrocytes. *J Histochem Cytochem*. 2003; 51(10):1285–1290. [PubMed: 14500696]
- Dickstein DL, et al. Changes in the structural complexity of the aged brain. *Aging Cell*. 2007; 6(3):275–284. [PubMed: 17465981]
- Douillard-Guilloux G, et al. Anticipated brain molecular aging in major depression. *Am J Geriatr Psychiatry*. 2013; 21(5):450–460. [PubMed: 23570888]
- Emmanuele V, et al. Decreased hippocampal expression of calbindin D28K and cognitive impairment in MELAS. *J Neurol Sci*. 2012; 317(1–2):29–34. [PubMed: 22483853]
- Erraji-Benchekroun L, et al. Molecular aging in human prefrontal cortex is selective and continuous throughout adult life. *Biological psychiatry*. 2005; 57(5):549–558. [PubMed: 15737671]
- Fishilevich S, et al. Genic insights from integrated human proteomics in GeneCards. Database (Oxford). 2016; 2016
- Fiske A, et al. Depression in older adults. *Annu Rev Clin Psychol*. 2009; 5:363–389. [PubMed: 19327033]
- Frisoni GB, et al. In vivo neuropathology of cortical changes in elderly persons with schizophrenia. *Biological psychiatry*. 2009; 66(6):578–585. [PubMed: 19409532]
- Fung SJ, et al. Expression of interneuron markers in the dorsolateral prefrontal cortex of the developing human and in schizophrenia. *The American journal of psychiatry*. 2010; 167(12):1479–1488. [PubMed: 21041246]
- Gaiteri C, et al. Beyond modules and hubs: the potential of gene coexpression networks for investigating molecular mechanisms of complex brain disorders. *Genes Brain Behav*. 2014; 13(1):13–24. [PubMed: 24320616]
- Geerligs L, et al. A Brain-Wide Study of Age-Related Changes in Functional Connectivity. *Cereb Cortex*. 2015; 25(7):1987–1999. [PubMed: 24532319]
- Glorioso C, Sibille E. Between destiny and disease: genetics and molecular pathways of human central nervous system aging. *Prog Neurobiol*. 2011; 93(2):165–181. [PubMed: 21130140]
- Grady C. The cognitive neuroscience of ageing. *Nature reviews Neuroscience*. 2012; 13(7):491–505. [PubMed: 22714020]
- He J, et al. Immunohistochemical staining, laser capture microdissection, and filter-aided sample preparation-assisted proteomic analysis of target cell populations within tissue samples. *Electrophoresis*. 2013; 34(11):1627–1636. [PubMed: 23436586]
- Hook VY, et al. Inhibitors of cathepsin B improve memory and reduce beta-amyloid in transgenic Alzheimer disease mice expressing the wild-type, but not the Swedish mutant, beta-secretase site of the amyloid precursor protein. *J Biol Chem*. 2008; 283(12):7745–7753. [PubMed: 18184658]

- Koenig HG, Blazer DG. Epidemiology of geriatric affective disorders. *Clin Geriatr Med*. 1992; 8(2): 235–251.
- Kringelbach ML. The human orbitofrontal cortex: linking reward to hedonic experience. *Nature reviews Neuroscience*. 2005; 6(9):691–702. [PubMed: 16136173]
- Lacerda AL, et al. Anatomic evaluation of the orbitofrontal cortex in major depressive disorder. *Biological psychiatry*. 2004; 55(4):353–358. [PubMed: 14960287]
- Lamar M, Resnick SM. Aging and prefrontal functions: dissociating orbitofrontal and dorsolateral abilities. *Neurobiol Aging*. 2004; 25(4):553–558. [PubMed: 15013577]
- Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008; 9:559. [PubMed: 19114008]
- Leal SL, Yassa MA. Neurocognitive Aging and the Hippocampus across Species. *Trends Neurosci*. 2015; 38(12):800–812. [PubMed: 26607684]
- Legon W, et al. Altered Prefrontal Excitation/Inhibition Balance and Prefrontal Output: Markers of Aging in Human Memory Networks. *Cereb Cortex*. 2015
- Lopez-Otin C, et al. The hallmarks of aging. *Cell*. 2013; 153(6):1194–1217. [PubMed: 23746838]
- Lu T, et al. Gene regulation and DNA damage in the ageing human brain. *Nature*. 2004; 429(6994): 883–891. [PubMed: 15190254]
- Lu T, et al. REST and stress resistance in ageing and Alzheimer's disease. *Nature*. 2014; 507(7493): 448–454. [PubMed: 24670762]
- Maciag D, et al. Reduced density of calbindin immunoreactive GABAergic neurons in the occipital cortex in major depression: relevance to neuroimaging studies. *Biological psychiatry*. 2010; 67(5): 465–470. [PubMed: 20004363]
- Manavalan A, et al. Brain site-specific proteome changes in aging-related dementia. *Exp Mol Med*. 2013; 45:e39. [PubMed: 24008896]
- McQuail JA, et al. Molecular aspects of age-related cognitive decline: the role of GABA signaling. *Trends Mol Med*. 2015; 21(7):450–460. [PubMed: 26070271]
- Morrison JH, Baxter MG. The ageing cortical synapse: hallmarks and implications for cognitive decline. *Nature reviews Neuroscience*. 2012; 13(4):240–250. [PubMed: 22395804]
- Northoff G, Sibille E. Why are cortical GABA neurons relevant to internal focus in depression? A cross-level model linking cellular, biochemical and neural network findings. *Molecular psychiatry*. 2014; 19(9):966–977. [PubMed: 25048001]
- Pan S, et al. Proteomics identification of proteins in human cortex using multidimensional separations and MALDI tandem mass spectrometer. *Molecular & cellular proteomics: MCP*. 2007; 6(10): 1818–1823. [PubMed: 17644759]
- Patel M. Targeting Oxidative Stress in Central Nervous System Disorders. *Trends Pharmacol Sci*. 2016; 37(9):768–778. [PubMed: 27491897]
- Resnick SM, et al. Vulnerability of the orbitofrontal cortex to age-associated structural and functional brain changes. *Annals of the New York Academy of Sciences*. 2007; 1121:562–575. [PubMed: 17846159]
- Rosso AL, et al. Aging, the central nervous system, and mobility. *J Gerontol A Biol Sci Med Sci*. 2013; 68(11):1379–1386. [PubMed: 23843270]
- Schwanhaussner B, et al. Global quantification of mammalian gene expression control. *Nature*. 2011; 473(7347):337–342. [PubMed: 21593866]
- Scifo E, et al. Proteomic analysis of the palmitoyl protein thioesterase 1 interactome in SH-SY5Y human neuroblastoma cells. *J Proteomics*. 2015; 123:42–53. [PubMed: 25865307]
- Shrestha P, et al. Layer 2/3 pyramidal cells in the medial prefrontal cortex moderate stress induced depressive behaviors. *Elife*. 2015:4.
- Sibille E. Molecular aging of the brain, neuroplasticity, and vulnerability to depression and other brain-related disorders. *Dialogues Clin Neurosci*. 2013; 15(1):53–65. [PubMed: 23576889]
- Silberberg D, et al. Brain and other nervous system disorders across the lifespan - global challenges and opportunities. *Nature*. 2015; 527(7578):S151–154. [PubMed: 26580320]
- Soreq L, et al. Major Shifts in Glial Regional Identity Are a Transcriptional Hallmark of Human Brain Aging. *Cell Rep*. 2017; 18(2):557–570. [PubMed: 28076797]

- Tondelli M, et al. Structural MRI changes detectable up to ten years before clinical Alzheimer's disease. *Neurobiol Aging*. 2012; 33(4):825, e825–836.
- Wang X, et al. Detecting disease-associated genes with confounding variable adjustment and the impact on genomic meta-analysis: with application to major depressive disorder. *BMC Bioinformatics*. 2012; 13:52. [PubMed: 22458711]
- Wisniewski JR. Proteomic sample preparation from formalin fixed and paraffin embedded tissue. *J Vis Exp*. 2013; (79)
- Xu B, et al. Quantitative protein profiling of hippocampus during human aging. *Neurobiol Aging*. 2016a; 39:46–56. [PubMed: 26923401]
- Xu B, et al. Temporal lobe in human aging: A quantitative protein profiling study of samples from Chinese Human Brain Bank. *Exp Gerontol*. 2016b; 73:31–41. [PubMed: 26631761]
- Yankner BA, et al. The aging brain. *Annu Rev Pathol*. 2008; 3:41–66. [PubMed: 18039130]
- Zhang W, et al. Comprehensive proteome analysis of fresh frozen and optimal cutting temperature (OCT) embedded primary non-small cell lung carcinoma by LC-MS/MS. *Methods*. 2015; 81:50–55. [PubMed: 25721092]
- Zubarev RA. The challenge of the proteome dynamic range and its implications for in-depth proteomics. *Proteomics*. 2013; 13(5):723–726. [PubMed: 23307342]

Highlights

- An unbiased proteomic study on postmortem OFC layer 2/3 during human aging
- Brain-specific representation of peripheral “hallmarks” of aging
- Alterations in neural communication and proteostasis “hallmarks” during aging
- Resilience in OFC layer 2/3 protein co-expression networks during aging
- Significant association of differential expressed proteins with age-related diseases

Figure 1. Proteomic changes in OFC layer 2/3 during human aging
(A) The histogram distribution of $\log_2$ (old/young) protein ratios for 4,193 proteins. (B) Heat map of differentially expressed proteins (127, 67 up- and 65 down-regulated proteins, RIM coefficient effect size ± 0.263 with a p -value < 0.05). Rows indicate proteins and columns indicate subjects. Color transition from red to green indicates upregulated and green to red indicates downregulated during aging. (C) Biological processes and disease categories identified by functional analysis using gorilla, g:Profiler and IPA on differentially expressed proteins.

Figure 2. Hallmarks of Aging-based categorization of differentially expressed proteins

Classification of OFC layer 2/3 differentially expressed 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 two most represented in OFC layer 2/3 differentially expressed proteins. None of the differentially expressed proteins were associated with telomere attrition. Green and red color protein symbols indicate up- and down-regulated during aging.

Figure 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 DEP (differentially expressed proteins) row demonstrates the distribution of either up- or down-regulated 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 represents the degree of connections. Red color represents the highest degree of connections (i.e., hubs)

and green color represents very few or lowest degree of connections. The distances between the nodes are arbitrary and have been modified in cytoscape for better visualization of the nodes and edges.

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Figure 4. RNA-Seq and Immunoblot validation of proteomic results

(A) A correlation plot between 1,555 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 IPA on differentially expressed genes. Several biological processes related to synaptic transmission, proteostasis, and cell development were commonly enriched in differentially expressed genes. Glutamate receptor signaling process was one of the significantly enriched pathways after IPA analysis on differentially expressed genes. IPA analysis also demonstrated a significant association between differentially expressed genes with several neurological 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).

Figure 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. Up – or down-regulated protein or gene symbols are indicated in green or red color. Italicized and underlined synaptic gene or protein symbols indicate those replicated from our previous reports (Douillard-Guilloux et al., 2013; Erraji-Benchekroun et al., 2005).

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. Grey 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 Zsummary	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