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Hippocampal gene expression patterns linked to late-life physical activity oppose age and AD-related transcriptional decline

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ABSTRACT

Exercise has emerged as a powerful variable that can improve cognitive function and delay age-associated cognitive decline and Alzheimer's disease (AD); however, the underlying mechanisms are poorly understood. To determine if protective mechanisms may occur at the transcriptional level, we used microarrays to investigate the relationship between physical activity levels and gene expression patterns in the cognitively intact aged human hippocampus. In parallel, hippocampal gene expression patterns associated with aging and AD were assessed using publicly available microarray data profiling hippocampus from young (20–59 years), cognitively intact aging (73–95 years) and age-matched AD cases. To identify "anti-aging/AD" transcription patterns associated with physical activity, probesets significantly associated with both physical activity and aging/AD were identified and their directions of expression change in each condition were compared. Remarkably, of the 2210 probesets significant in both data sets, nearly 95% showed opposite transcription patterns with physical activity compared with aging/AD. The majority (>70%) of these anti-aging/AD genes showed increased expression with physical activity and decreased expression in aging/AD. Enrichment analysis of the anti-aging/AD genes showing increased expression in association with physical activity revealed strong overrepresentation of mitochondrial energy production and synaptic function, along with axonal function and myelin integrity. Synaptic genes were notably enriched for synaptic vesicle priming, release and recycling, glutamate and GABA signaling, and spine plasticity. Anti-aging/AD genes showing decreased expression in association with physical activity were enriched for transcription-related function (notably negative regulation of transcription). These data reveal that physical activity is associated with a more youthful profile in the hippocampus across multiple biological processes, providing a potential molecular foundation for how physical activity can delay age- and AD-related decline of hippocampal function.
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1. Introduction

Lifestyle factors have a striking ability to empower the adult brain—strengthening plasticity, building cognitive reserve, and preventing functional decline. Physical activity in particular has emerged as a powerful life-style factor that can improve cognitive function (Buchman et al., 2008, 2012; Colcombe and Kramer, 2003; Colcombe et al., 2003; Erickson et al., 2010, 2011; Kramer and Erickson, 2007a,b; Smith et al., 2010; Stubbs et al., 2017; Suwabe et al., 2018), reduce the risk of age-associated cognitive decline and Alzheimer's disease (AD) (Buchman et al., 2012; Erickson et al., 2012; Hillman et al., 2008; Kirk-Sanchez and McGough, 2014; Korol et al., 2013; Prakash et al., 2015; Weuve et al., 2004; Yaffe et al., 2001), counteract age- and AD-related losses of gray and white matter (Baker et al., 2010; Best et al., 2017; Bherer et al., 2013; Colcombe et al., 2003; Erickson et al., 2014; Lautenschlager et al., 2008; Okonkwo et al., 2014; Suzuki et al., 2012; Voss et al., 2013; Zlatar et al., 2015), and counteract age- and AD-related declines in cognitive neural network function (Colcombe et al., 2004; Huang et al., 2016; Voss et al., 2010). Although the benefits of physical activity are evident, the underlying mechanisms by which physical activity supports brain function and health and slows aging- and AD-related declines in the human brain are poorly understood.

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Gene expression studies to address this question have been precluded by a paucity of human autopsy cases that are well-defined for late-life lifestyle factors, and in particular, where physical activity has been objectively documented in a manner that avoids recall bias. Participants in the Rush Memory and Aging Project (MAP) meet these criteria. MAP participants have been followed longitudinally for up to 18 years, until death, with detailed annual assessments of cognitive function and multiple lifestyle variables including quantitative assessment of physical activity (Bennett et al., 2012). Importantly, physical activity was continuously monitored over multiple days with actigraphy, capturing both exercise and non-exercise activity (e.g., light physical activity) to provide a highly accurate account of the total daily activity levels (Buchman et al., 2012).

In this study, we use post-mortem hippocampal tissue from cognitively healthy, aged (76–100 years) MAP cases that captured a broad spectrum of physical activity level in combination with a microarray-based genome-wide approach to investigate mechanisms by which physical activity may promote cognitive health and hippocampal function and counteract aging- and AD-related transcription patterns in the human brain. The data suggest that gene expression patterns associated with physical activity have the potential to extensively counteract transcriptional changes that occur in the human hippocampus with aging or AD. In particular, physical activity may slow aging-/AD-related declines in hippocampal health and function by enhancing mitochondrial energy production and synaptic function, improving axonal function and myelin integrity and maintaining appropriate transcriptional control mechanisms.

2. Materials and methods

2.1. Samples

To investigate the relationship between physical activity and gene expression patterns in the cognitively intact aged human hippocampus, frozen post-mortem hippocampal brain tissue was obtained from 47 clinically well-characterized cognitively intact cases (aged 76–100 years) from MAP, an ongoing longitudinal, study of older individuals without known dementia recruited from northeastern Illinois. All tissues were obtained from the mid-hippocampus region containing all hippocampal subfields. Participants were diagnosed as cognitively intact based on annual assessment of cognitive status using a battery of 19 cognitive tests, at which time they were additionally assessed for other lifestyle factors including cognitive frequency, social frequency, and depression, among other variables (Bennett et al., 2012). Participants underwent annual assessment of physical activity using actigraphs worn continuously over multiple days. Postmortem tissue was assessed for pathology as previously reported (Bennett et al., 2012), and cases with Lewy Bodies, infarcts, or hippocampal sclerosis were excluded from the study. Hippocampal RNA from 35 participants (76–100 years, average 87.62 $\pm$ 6.36 years) was of high quality sufficient to process on microarrays and an additional 12 samples were included in the Nanostring analysis.

2.2. Quantification of physical activity

Physical activity was measured with actigraphy (Actical; MiniMitter, Bend, OR, USA). Total daily exercise and non-exercise physical activity were measured 24 hours per day for up to 10 days, with actigraphs worn on the nondominant wrist. Average daily physical activity was calculated as the average across days of the total daily activity counts recorded. Average physical activity ranged from 0.13 to 5.6 $\times$ 10^5 activity counts/d (Supplemental Fig. 1). Data were converted to approximate metabolic equivalents (METs), using the distance conversion metrics reported by Hall et al., 2013 and standard MET equivalent values (Ainsworth et al., 1993). Using these metrics, 1.0 $\times$ 10^5 counts/d corresponded to 20 min/d moderate-high activity (walking, 3.5 mph) (equivalent to 1.5 MET hours of activity/d), and 5.0 $\times$ 10^5 counts/d was the equivalent of 100 min/d of moderate-high activity (7.5 MET-h/d). Previously, Weuve et al., 2004, described a dose-dependent effect of physical activity on cognitive performance, with highest cognitive performance in individuals with activity estimates above 26 MET h/wk.

2.3. RNA extraction and microarray quality control

RNA from approximately 25 mg tissue (mid-level hippocampus containing all hippocampal subregions) was extracted using Trizol, treated with DNAse, and purified using Qiagen spin columns as described previously (Berchtold et al., 2008). RNA quality was assessed using the Agilent Bioanalyzer and RNA integrity number (RIN), and samples with RIN $\geq$6.5 were further processed on microarrays. CEL file quality control was assessed using the affyQC automated R workflow available through ArrayAnalysis (http://www.arrayanalysis.org). Quality control parameters were beta actin 3'/5' ratio $<$3, GAPDH 3'/5' ratio $<$1.25, internal hybridization control signal (BioB = Present), Percent Present $\geq$49%, Percent Present spread $\leq$10%, Background spread $\leq$20%) and log scale factor spread $\leq$3.

2.3.1. Actical samples

Of the 47 hippocampal tissues, high-quality RNA was obtained from 35 samples (RIN: 7.36 $\pm$ 0.58, range 6.5–8.7), which were processed individually on Affymetrix HgU133plus 2.0 microarrays by the Genomics High-Throughput Facility at UC Irvine, following manufacturer's recommendations. CEL file quality control was assessed using the affyQC workflow in ArrayAnalysis (http://www.arrayanalysis.org). One microarray did not meet all quality control parameters in ArrayAnalysis and was excluded. Sample characteristics and quality control measures are provided in Supplemental Tables 1A and 1D. The remaining 34 CEL files were submitted to GeneSpring software GS version 14.5 (Agilent Technologies) for preprocessing by MicroAarray Suite v.5 (MAS) and GC-robust multi-array average (GC-RMA). MAS and GC-RMA values were quantile normalized and underwent baseline transformation to the median of all samples across all genes as implemented in GS version 14.5. The CEL files and microarray data are available through the public gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) and data set accession number GSE110298.

2.3.2. Young, aged, and AD samples

To characterize gene expression changes associated with aging and AD, 58 CEL files profiling gene expression patterns in the hippocampus from young (20–59 years), cognitively intact aging cases (69–99 years), and AD cases (73–99 years) were downloaded from the public GEO (http://www.ncbi.nlm.nih.gov/geo) data set and accession numbers GSE11882 and GSE48350. CEL file quality control was assessed using the affyQC workflow in ArrayAnalysis (http://www.arrayanalysis.org) and identified 48 microarrays that met all quality control and study parameters [young (n = 16), cognitively intact aging cases (n = 18), and AD cases (n = 14)]. Sample characteristics and quality control measures are provided in Supplemental Tables 1B and 1E.

2.4. Microarray data analysis—identifying genes associated with physical activity

Poorly performing probesets were eliminated by filtering the array data based on flags (Present, Marginal, Absent) using MAS preprocessing, retaining only probesets flagged present on all samples of at least one physical activity group (low, moderate, high

activity: Supplemental Fig. 1) (23,712 probesets) and that overlapped with the probeset list used for the analysis of aging and AD (21,002 probesets). Using the programming environment R (http://www.R-project.org/), multiple linear regression was applied to GC-RMA–normalized values using lm(·) to adjust for technical factors that were potential confounding variables (batch, RIN, and post-mortem interval) and to evaluate the relationship between gene expression and late-life physical activity levels across the 34 samples (Miller et al., 2017). For each probeset, the estimated regression coefficient for physical activity, its standard error, and the t-ratio and p-value for a two-sided test were extracted using infer(·) with robust standard errors. Significance was set at $p \leq 0.05$, and q values were calculated with GraphPad Prism 7 statistical software using the two-stage step-up method of Benjamini, Krieger, and Yekutieli. To estimate fold changes across low ($\leq 1.0 \times 10^5$ counts/d), moderate (1.0×10^5–2.0×10^5 counts/d), and high physical activity ($>2.1 \times 10^5$ counts/d) tiers, multiple linear regression was applied to each 2 group comparison, and p and q values were calculated. Log values were transformed to linear scale using Excel software. All calculated values are provided in Supplemental Table 2.

2.5. Microarray analysis–identifying genes associated with aging and AD

The 54,675 probesets on the array were filtered using MAS preprocessing–derived flags to retain only probesets flagged present on all samples of at least one treatment group (young, aged, or AD) (21,002 probesets) and that overlapped with the probeset list used for the analysis of gene expression associated with physical activity (21,002 probesets). Using R, multiple linear regression was applied to GC-RMA normalized values to adjust for batch, RIN, and post-mortem interval and to evaluate gene expression changes in aging (aged vs. young) and AD (AD vs. aged). For each probeset, the estimated regression coefficient, its standard error, and the t-ratio and p-value for a two-sided test were extracted using infer() with robust standard errors. Significance was set at $p \leq 0.05$, and q values were calculated using GraphPad Prism 7 statistical software. Log values were transformed to linear scale using Excel software. All calculated values are provided in Supplemental Table 2.

2.6. Anti-aging/AD patterns associated with exercise

To investigate mechanisms by which physical activity may protect the brain from aging- and AD-related change, probesets significantly associated with both physical activity and aging or AD were selected, patterns of expression change were compared, and probesets showing opposite patterns of change with physical activity versus aging or AD were identified (henceforth referred to as "anti-aging/AD probesets") (see Methods in Fig. 1). Significance of the overlap patterns between physical activity and aging-AD (e.g., opposite direction of gene expression change, or similar direction) was assessed using the Fisher exact test. 2 approaches were used to estimate the number of probesets expected to occur by chance in the comparisons of physical activity and aging, or physical activity and AD. The first approach assumes normality of the p value distribution and independence of the comparisons and was calculated as $(2^*(0.05^2)–(0.05)^3)$ * 21,002 probesets tested. This approach estimated the number of putative false positives to be 102 probesets (e.g., $p \leq 0.004875 \times 21{,}002$), corresponding to a false positive rate (FDR) estimate of 4.6% (e.g., 102 false discoveries/2210 true discoveries). Permutation analysis was used as a second approach where normality of the p value distribution is not assumed. Using the R function "transform(·)," activity labels were randomized across samples in the physical activity data set, and across the young, aged, and AD samples for the aging-AD analysis, and the number of overlapping significant probes was calculated for the permuted data. One hundred permutation analyses were performed, resulting in estimations ranging from 10 to 1057 false positives, with an average ± standard deviation estimate of 176 ± 192 and a median estimate of 109 false positives. The computed average false positives (176) corresponds to an FDR of 7.9%, and the median false positive estimate (109) corresponds to an FDR of 4.9%, similar to the FDR estimate (4.6%) calculated under the assumption of normal distribution of p values.

2.7. DAVID functional enrichment analysis

Functional enrichment analysis of the Anti-aging/AD genes was used to identify biological modules of aging/AD that show the opposite pattern of change with physical activity. Significant gene lists were submitted to the high-throughput data mining bioinformatics resource the Database for Annotation, Visualization and Integrated Discovery (DAVID: https://david.ncifcrf.gov) (Dennis et al., 2003; Huang da et al., 2009a,b) to identify over-represented biological functions and pathways. Significant probesets were translated to DAVID ids followed by mapping of unambiguous DAVID ids to terms in the Gene Ontology (GO) databases [Cell Component (GOTERM-CC), Biological Process (GOTERM-BP), Molecular Function (GOTERM-MF)], Kyoto Encyclopedia of Genes and Genomes (KEGG) and BioCarta pathways, or the UniProt keywords database. Enrichment was assessed against a background gene set of the 21,002 probesets with reliable expression on both data sets, Expression Analysis Systematic Explorer score threshold $p \leq 0.01$, and Benjamini multiple testing correction $p \leq 0.10$. Benjamini p-values are indicated in the results.

2.8. Nanostring nCounter gene expression system

Microarray expression changes associated with physical activity were validated using the NanoString nCounter gene expression system to assess gene expression on an expanded set of human hippocampal tissues, for experimental and independent replication of the microarray data. Experimental replication included 8 additional low activity cases and 4 additional high physical activity cases, in addition to low (n = 10) and high activity (n = 12) cases included in the microarray analysis (Supplemental Tables 1C, 1D). One low activity sample (sample 1_1-E) included in the microarray analysis was not included in the nanostring run because of sample degradation. NanoString provides similar sensitivity to TaqMan-based RQ-PCR and SYBR Green I fluorescent dye–based RQ-PCR but does not involve reverse transcription of mRNA and subsequent cDNA amplification, thus eliminating amplification bias (Geiss et al., 2008; Veldman-Jones et al., 2015). Gene expression was assessed in low activity (n = 18) and high activity (n = 16) cases for 13 synaptic genes that showed decreased expression with aging/AD and increased expression with physical activity (GABRG2, glycine receptor beta [GLRB], GRIN2a, SLC1a6, synapsin II [SYN2], VAMP2, synaptosome-associated protein 25 [SNAP25], USP14, NRXN1, NRXN3, SYT4, somatostatin receptor 1 [SSTR1], Munc13-3), as well as for 10 genes where microarray analysis demonstrated significantly decreased expression with aging/AD but only trends ($p < 0.1$) for increased expression with physical activity (GABBR1, GABRA2, GABRA4, GABRB3, GABRG1, GRIA2, DOC2a, SCN2b, DLG3/SAP102, AKAP5). Seven putative internal control genes (ATF6, DDX52, METTL20, GAPDH, SLC38a7, ZNF780b, ZNF791) were first identified based on microarray data demonstrating expression homogeneity across physical activity tier and low variance and were evaluated using NanoString for suitability. Putative control genes were excluded if NanoString gene expression showed a trend ($p \leq 0.1$) for a significant correlation with physical activity across cases or a trend for a significant t-test comparing low versus high

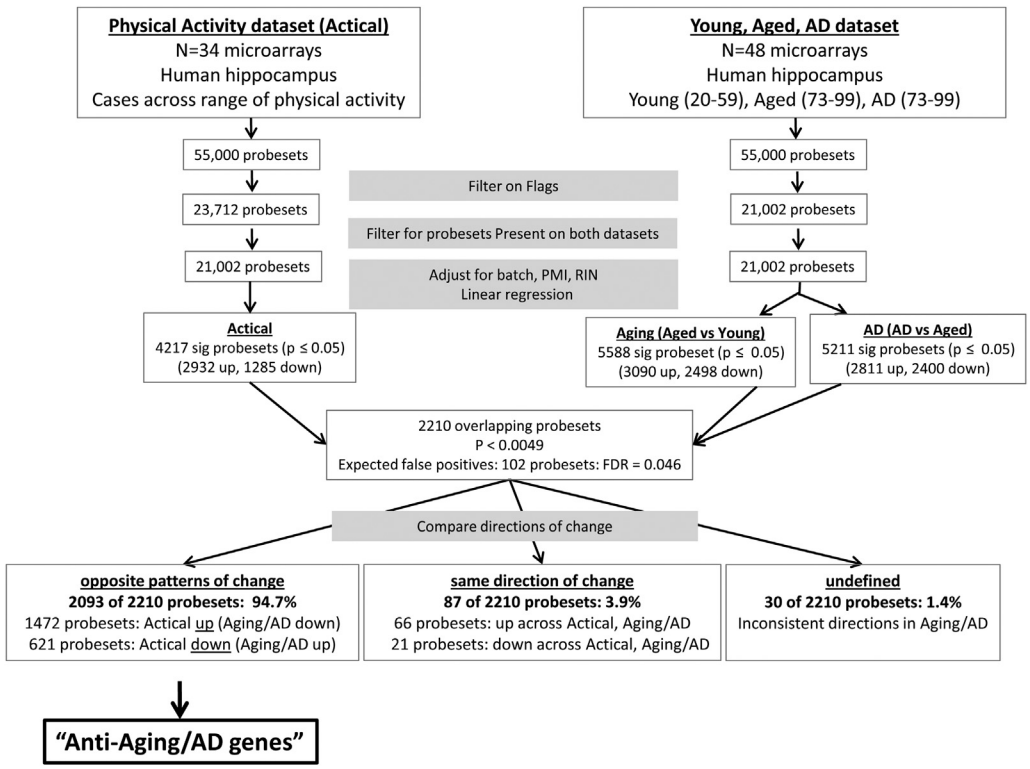

Fig. 1. Flowchart of the methods to determine if physical activity–related gene expression patterns counter aging and AD-related expression change. Thirty four microarrays profiling gene expression in the aged human hippocampus were used to determine the relationship between gene expression and physical activity. Poorly performing probesets were eliminated by filtering the array data based on flags (present, marginal, absent) using MAS preprocessing, retaining only probesets flagged present on all samples of at least one group (low, moderate, high activity) (23,034 probesets) and that also met these criteria in the aged-AD analysis (21,002 probesets). Using the programming environment R (http://www.R-project.org/), multiple linear regression was applied to GC-RMA–normalized values using lm(·) to adjust for technical factors that were potential confounding variables (batch, RIN, and PMI) and to evaluate the relationship between gene expression and late-life physical activity levels across the 34 samples. In parallel, aging and AD-related gene expression change was assessed using 51 CEL files from the public gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo, data set accession number GSE: 11882) profiling expression patterns in hippocampal samples from young cases (20–59 years, n = 17), cognitively intact aged cases (73–99 years, n = 20), and AD cases (73–99 years, n = 14). The ~55,000 probesets on the array were filtered using MAS preprocessing-derived flags to retain only probesets flagged present on all samples of at least one treatment group (21,002 probesets) and that also met these criteria in the physical activity analysis (21,002 probesetts). Using R, multiple linear regression was applied to GC-RMA–normalized values to adjust for batch, RIN, and PMI and to evaluate gene expression changes in aging (aging vs. young) and AD (AD vs. aged). Probesets significantly associated with both physical activity ($p < 0.05$) and aging ($p < 0.05$) or AD ($p < 0.05$) were selected, patterns of expression change were compared for each probeset, and probesets showing opposite patterns of change with physical activity versus aging or AD were identified. The analysis identified 2138 probesets significantly associated with both physical activity and aging or AD, substantially more than the number of probesets expected to occur by chance in the overlap of the 2 studies (102 probesets), for a false discovery rate (FDR) = 4.6%. Of the 2138 significant overlapping probesets, 94.9% (2029 probesets) of which showed the opposite pattern of change with physical activity compared to aging/AD ("anti-aging/AD probesets"). These probesets were analyzed for functional enrichment using DAVID. Abbreviations: AD, Alzheimer's disease; GC-RMA, GC-robust multi-array average; MAS, MicroAarray Suite v.5; RIN, RNA integrity number; PMI, post-mortem interval; DAVID, Database for Annotation, Visualization and Integrated Discovery.

physical activity groups. ZNF780b and DDX52 were identified as suitable control genes. Nanostring expression values underwent sample normalization to the geometric mean of the control genes and Excel software was used to calculate means, standard error of the mean (sem), and 2 group comparisons (t-tests) of the low activity (n = 18) and high activity (n = 16) groups.

For independent validation of the microarray data, only the new NanoString cases were included in the analysis (n = 8 low activity, n = 4 high activity). The Mann-Whitney test was used to determine whether the NanoString-measured genes, as a group, tended to move in the same direction as those genes did in the microarray analysis. For each gene, the average NanoString value for the low and high activity groups were calculated, and the Mann-Whitney test was applied using GraphPad Prism 7 statistical software.

3. Results

3.1. Physical activity is associated with anti-aging/AD gene expression patterns in the hippocampus

Hippocampal tissue from 34 cognitively intact aged cases well characterized for physical activity levels were run on microarrays to investigate gene expression patterns significantly associated with physical activity levels. In parallel, microarray data profiling hippocampal gene signatures from young (20–59 years), cognitively intact aging (69–99 years) and AD (73–99) cases were analyzed to identify gene expression patterns associated with aging and AD (GEO data set accession number GSE11882, http://www.ncbi.nlm.nih.gov/geo). To investigate mechanisms by which physical activity may protect the brain from aging and AD-related change, probesets significantly associated with both physical activity and aging or AD were selected, patterns of expression change were compared for each probeset, and probesets showing opposite patterns of change with physical activity versus aging or AD were identified (henceforth referred to as "anti-aging/AD probesets") (Fig. 1).

Of the 21,002 probesets with reliable expression on both data sets, the analysis identified 2210 probesets that were significantly associated with both physical activity and aging/AD, a number far greater than expected to occur by chance (e.g., ~102 expected false positives/2210 significant probesets, FDR = 4.6%), with the significance of the overlap calculated to be $p < 0.0001$ (Fisher exact test). Remarkably, of these 2210 probesets, 94.7% (2093 probesets) showed the opposite patterns of change in association with physical activity compared with aging/AD. The majority (>70%) of these anti-aging/

AD probesets showed increased expression with physical activity (1472 probesets), whereas ~30% (621 probesets) showed decreased expression in association with physical activity. These data suggest that gene expression patterns associated with physical activity extensively counteract gene expression changes that occur in the human hippocampus with aging or AD, particularly with respect to those genes undergoing transcriptional decline.

3.2. Anti-aging/AD genes are enriched for several core biological functions

To investigate potential repercussions of physical activity for hippocampal health and function, we investigated if the anti-aging/AD probesets significantly associated with physical activity were enriched for biological functions using the integrated data mining bioinformatics resource DAVID version 6.8 (Dennis et al., 2003; Huang da et al., 2009a,b). The anti-aging/AD probeset lists (1472 with increased expression and 621 with decreased expression in association with physical activity) were uploaded and translated to DAVID ids followed by mapping of unambiguous DAVID ids to terms in the GO databases, KEGG pathways, or UniProt keywords database. Benjamini *p*-values are indicated throughout the results.

For anti-aging/AD probesets showing increased expression in association with physical activity, the probeset list translated into 1176 unique and unambiguous DAVID ids of which 1117 genes could be mapped to database terms. DAVID analysis revealed robust enrichment for several functional categories key to cellular health and synaptic function (Table 1). Highly significant enrichment was found for mitochondria-related genes (e.g., UniProt keyword "Mitochondrion," $p = 2.0 \times 10^{-5}$), especially for electron transport chain function, as well as for genes associated with the synapse (e.g., UniProt keyword "Synapse," $p = 5.1 \times 10^{-7}$), myelin sheath (GOTERM "myelin sheath," $p = 1.0 \times 10^{-4}$), and axon (GOTERM "axon," $p = 3.4 \times 10^{-3}$). These data suggest that aging/AD is accompanied by diminishing gene expression for these core biological functions, and that physical activity may slow aging/AD-related declines in hippocampal health and function by enhancing mitochondrial energy production, synaptic health, and axon function.

A different enrichment pattern was observed for anti-aging/AD probesets showing decreased expression in association with physical activity. These 621 anti-aging/AD translated into 408 unique and unambiguous DAVID ids of which 378 genes could be mapped to an enrichment term. Anti-aging/AD genes showing decreased expression with physical activity were significantly enriched primarily for transcription-related function (e.g., UniProt keyword "Transcription," $p = 7.1 \times 10^{-3}$), notably for negative regulation of transcription (e.g., GOTERM "negative regulation of transcription from RNA polymerase II promoter", $p = 8.8 \times 10^{-2}$) (Table 2). These data suggest that aging/AD are accompanied by increased expression of genes that suppress transcription, and that physical activity may restrain this transcriptional dysregulation to maintain appropriate transcriptional control of gene expression in the aging and AD hippocampus.

3.2.1. Physical activity–associated gene expression patterns oppose aging/AD-related declines in mitochondrial function and energy production

DAVID analysis revealed that among anti-aging/AD genes increased with physical activity, mitochondria-related genes were the most highly enriched class and represented 12%–13% of the gene list, a 1.5-fold enrichment (GOTERM-CC, $p = 7.0 \times 10^{-6}$). These included pronounced enrichment for oxidative phosphorylation (KEGG pathway, 3.17-fold enrichment, $p = 5.5 \times 10^{-7}$) along with components of the mitochondrial inner membrane (UniProt keyword: 2.3-fold enrichment, $p = 4.4 \times 10^{-7}$) and mitochondrial outer membrane (UniProt keyword: 2.1-fold enrichment, $p = 9.6 \times 10^{-2}$). These mitochondria-associated genes were next investigated in greater detail to determine which aspects of mitochondrial function that decline with aging/AD may be particularly amenable to improvement with physical activity. Genes in these enriched mitochondria-related categories have been compiled in Supplemental Table 3.

Approximately 30% of the mitochondrial anti-aging/AD genes were associated with energy production via oxidative phosphorylation and the tricarboxylic acid (TCA) cycle. Anti-aging/AD genes were 3.4-fold enriched for cellular components of the mitochondrial respiratory chain complex I (GOTERM-CC, $p = 4.8 \times 10^{-3}$) and 4.0-fold enriched for mitochondrial respiratory chain complex V (ATP synthase complex) (GOTERM-CC, $p = 9.7 \times 10^{-2}$). Overlay of all genes significantly associated with physical activity onto the KEGG pathway "oxidative phosphorylation" (3.2-fold enriched, $p = 5.5 \times 10^{-7}$) reveals the extensive engagement of genes critical for energy production. Increased expression was found for approximately 30% of the genes encoding mitochondrial complex I—the entry point to the electron transport pathway—along with 35% of the genes encoding complex IV (cytochrome C oxidase) and complex V (ATP synthase), as well as 2 components (SLC25A27, SLC25A14) of the uncoupling protein (Fig. 2). The broadly increased expression of oxidative phosphorylation genes was accompanied by increased expression of key genes driving pyruvate metabolism and the TCA cycle, which generates energy from pyruvate metabolism and provides NADH and FADH2 reducing equivalents for the electron transport chain. These included dihydrolipoamide dehydrogenase, malate dehydrogenase 1, malate dehydrogenase 2, malic enzyme 1, malic enzyme 3, pyruvate dehydrogenase, and succinate CoA ligase (beta subunit, SUCLA2). Other notable mitochondrial anti-aging/AD genes showing increased expression in association with physical activity included several mitochondrial ribosomal structural genes (MRPL1, MRPL14, MRPL15, MRPL30, MRPL35, MRPL49, MRPX16, MRPS21, MRPS22, MRPS25, MRPS26) (Supplemental Table 3)).

Taken together, these data suggest that multiple components necessary for mitochondrial health and energy production undergo declining gene expression with aging/AD, and that physical activity may slow aging/AD-related declines in hippocampal function by counteracting these gene expression patterns to enhance mitochondrial health and capacity for energy production.

3.2.2. Physical activity–associated gene expression patterns oppose aging/AD-related declines in synaptic gene expression

Genes related to synaptic and neuronal function constituted the second most significantly enriched class of genes among anti-aging/AD genes increased with physical activity. For example, genes in the Uniprot Keyword category "Synapse" represented ~5% of the gene list (60 genes), a 2.3-fold enrichment ($p = 5.1 \times 10^{-7}$) (Table 1). Significant genes related to synaptic function could be grouped into the following main functional categories: (1) synaptic vesicle priming, release, and recycling; (2) glutamate and GABA signaling and receptor trafficking; and (3) synapse formation, plasticity, and axon health. Significant genes in enriched terms related to synapse (Uniprot Keyword, $p = 5.1 \times 10^{-7}$), synaptic vesicle membrane (GOTERM-CC, 3.9-fold enriched, $p = 4.9 \times 10^{-4}$), chemical synaptic transmission (GOTERM-BP, 2.2-fold enriched, $p = 9.4 \times 10^{-2}$) and postsynaptic membrane (GOTERM-CC, 2.1-fold enriched, $p = 1.4 \times 10^{-2}$) are listed in Supplemental Table 4.

3.2.2.1. Synaptic vesicle priming, release, and recycling. Our data revealed that numerous anti-aging/AD genes showing increased activity with physical activity were associated with synaptic vesicle priming, regulating the readily releasable and reserve pools of synaptic vesicles, and synaptic vesicle recycling and recovering

Table 1
DAVID enrichment categories for unambiguous probesets with hippocampal expression significantly increased in association with physical activity and significantly decreased with aging or AD

Category	Database	Term	Count	%	EASE *p* value	List total	Pop hits	Pop total	Fold enrichment	Benjamini
Mitochondria-related categories										
Mitochondrion	UP_KEYWORDS		143	12.16	1.98E-07	1138	881	10,612	1.51	2.04E-05
Mitochondrion	GOTERM_CC_DIRECT	GO:0005739	162	13.78	3.57E-08	1047	1026	9961	1.50	7.04E-06
Mitochondrion inner membrane	UP_KEYWORDS		55	4.68	2.12E-09	1138	218	10,612	2.35	4.39E-07
Mitochondrion inner membrane	GOTERM_CC_DIRECT	GO:0005743	74	6.29	2.77E-08	1047	364	9961	1.93	8.20E-06
Mitochondrion outer membrane	UP_KEYWORDS		19	1.62	3.41E-03	1138	85	10,612	2.08	9.60E-02
Respiratory chain	UP_KEYWORDS		17	1.45	1.41E-04	1138	55	10,612	2.88	8.26E-03
Oxidative phosphorylation	KEGG_PATHWAY	hsa00190	33	2.81	2.19E-09	380	107	3909	3.17	5.54E-07
Mitochondrial respiratory chain complex I	GOTERM_CC_DIRECT	GO:0005747	15	1.28	5.70E-05	1047	42	9961	3.40	4.81E-03
Ubiquinone	UP_KEYWORDS		10	0.85	1.88E-03	1138	28	10,612	3.33	6.83E-02
Mitochondrial electron transport, NADH to ubiquinone	GOTERM_BP_DIRECT	GO:0006120	15	1.28	6.51E-05	972	43	9380	3.37	9.24E-02
Mitochondrial proton-transporting ATP synthase complex	GOTERM_CC_DIRECT	GO:0005753	8	0.68	2.23E-03	1047	19	9961	4.01	9.67E-02
Metabolic pathways	KEGG_PATHWAY	hsa01100	103	8.76	6.18E-04	380	792	3909	1.34	2.21E-02
Synapse, plasticity-related categories										
Synapse	UP_KEYWORDS		60	5.10	1.23E-09	1138	245	10,612	2.28	5.06E-07
Synaptic vesicle membrane	GOTERM_CC_DIRECT	GO:0030672	16	1.36	4.16E-06	1047	39	9961	3.90	4.92E-04
Chemical synaptic transmission	GOTERM_BP_DIRECT	GO:0007268	31	2.64	3.31E-05	972	134	9380	2.23	9.38E-02
Postsynaptic cell membrane	UP_KEYWORDS		26	2.21	1.46E-04	1138	108	10,612	2.24	7.50E-03
Postsynaptic membrane	GOTERM_CC_DIRECT	GO:0045211	29	2.47	2.08E-04	1047	132	9961	2.09	1.36E-02
CREB pathway: Transcription factor CREB and its extracellular signals	BIOCARTA		9	0.77	1.89E-03	102	26	1060	3.60	6.89E-02
Axon	GOTERM_CC_DIRECT	GO:0030424	38	3.23	3.40E-05	1047	178	9961	2.03	3.35E-03
Myelin sheath	GOTERM_CC_DIRECT	GO:0043209	36	3.06	7.04E-07	1047	140	9961	2.45	1.04E-04
Growth cone	GOTERM_CC_DIRECT	GO:0030426	23	1.96	1.62E-04	1047	92	9961	2.38	1.19E-02
Neuron projection	GOTERM_CC_DIRECT	GO:0043005	31	2.64	1.87E-03	1047	165	9961	1.79	9.56E-02
Cell junction	UP_KEYWORDS		78	6.63	4.37E-06	1138	433	10,612	1.68	3.61E-04
Cell junction	GOTERM_CC_DIRECT		66	5.61	2.16E-08	1047	308	9961	2.04	1.28E-05
Other categories										
Gap junction	KEGG_PATHWAY	hsa04540	16	1.36	1.25E-03	380	66	3909	2.49	3.12E-02
Circadian entrainment	KEGG_PATHWAY	hsa04713	17	1.45	6.86E-04	380	69	3909	2.53	2.15E-02
Transport	UP_KEYWORDS		172	14.63	4.71E-04	1138	1265	10,612	1.27	2.14E-02
Transit peptide	UP_KEYWORDS		77	6.55	1.07E-05	1138	436	10,612	1.65	7.33E-04
ChREBP pathway: ChREBP regulation by carbohydrates and cAMP	BIOCARTA		9	0.77	3.51E-05	102	16	1060	5.85	6.62E-03
Agpcr pathway: Attenuation of GPCR Signaling	BIOCARTA		7	0.60	2.02E-04	102	11	1060	6.61	1.89E-02
Csk pathway: Activation of Csk by cAMP-dependent protein kinase inhibits signaling through the T-cell receptor	BIOCARTA		7	0.60	1.03E-03	102	14	1060	5.20	6.30E-02
PLCe pathway: Phospholipase C-epsilon pathway	BIOCARTA		6	0.51	1.22E-03	102	10	1060	6.24	5.60E-02
Racc pathway: Ion channels and their functional role in vascular endothelium	BIOCARTA		6	0.51	2.07E-03	102	11	1060	5.67	6.31E-02
Nos1 pathway: Nitric oxide signaling pathway	BIOCARTA		7	0.60	2.35E-03	102	16	1060	4.55	6.15E-02
Gpcr pathway: Signaling pathway from G-protein families	BIOCARTA		9	0.77	3.19E-03	102	28	1060	3.34	7.28E-02

Note that significant genes may be contained within multiple enriched terms within a category.
Key: AD, Alzheimer's disease; EASE, Expression Analysis Systematic Explorer; DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(Table 1). Key enriched terms associated with these functions included synaptic vesicle membrane (GOTERM-CC, 3.9-fold enriched, $p = 4.9 \times 10^{-4}$) and chemical synaptic transmission (GOTERM-BP, $p = 9.4 \times 10^{-2}$).

Several key genes that regulate synaptic vesicle priming and docking at the presynaptic active zone were among the anti-aging/AD genes showing increased expression with physical activity (Supplemental Table 4). Synaptic vesicle priming and docking require the assembly of 3 essential SNARE proteins into a complex, followed by binding of the SNARE complex to Sec1/Munc18-like (SM) proteins and other chaperones to catalyze vesicle fusion to the presynaptic membrane. Our data revealed that anti-aging/AD genes included 2 of the 3 core SNARE proteins (SNAP25, synaptobrevin/VAMP2) that are essential for active zone priming and docking, as well as several SNARE chaperones that included syntaxin-binding proteins (syntaxin-binding protein 1/Munc18-1, syntaxin-binding protein 5), amyloid beta precursor protein, the RIM-binding protein ERC2, alpha and beta synuclein (SNCA, SNCB), and rab-3 interacting molecules (RIMS2, RIMS3).

In parallel, many of the anti-aging/AD genes increased with physical activity are key regulators of presynaptic vesicle availability. For example, among the anti-aging/AD genes were several active zone scaffolding proteins that regulate the balance between the reserve and the readily releasable pools of neurotransmitter, including SYN2, cyclin-dependent kinase 5, and syntaxin-binding protein 5. In addition, there were numerous genes that regulate endocytosis and recovery of synaptic vesicles after neurotransmitter release, highly important steps for maintaining presynaptic

Table 2
DAVID enrichment results for unambiguous probesets with hippocampal expression significantly decreased in association with physical activity and significantly increased with aging or AD

Category	Database	Term	Count	%	EASE *p* value	List total	Pop hits	Pop total	Fold enrichment	Benjamini
Nucleus	UP_KEYWORDS		155	37.99	3.08E-04	383	3398	10,612	1.26	1.93E-02
Transcription	UP_KEYWORDS		81	19.85	9.04E-05	383	1480	10,612	1.52	7.14E-03
Transcription regulation	UP_KEYWORDS		79	19.36	8.03E-05	383	1428	10,612	1.53	8.45E-03
Negative regulation of transcription from RNA polymerase II promoter	GOTERM_BP	GO:0000122	35	8.58	4.93E-05	357	436	9380	2.11	8.80E-02
Repressor	UP_KEYWORDS		31	7.60	3.64E-04	383	428	10,612	2.01	1.91E-02
Activator	UP_KEYWORDS		30	7.35	1.13E-03	383	438	10,612	1.90	4.37E-02
Polymorphism	UP_KEYWORDS		274	67.16	4.03E-08	383	6188	10,612	1.23	1.28E-05
Alternative splicing	UP_KEYWORDS		269	65.93	9.26E-04	383	6639	10,612	1.12	4.11E-02
Isopeptide bond	UP_KEYWORDS		48	11.76	1.94E-03	383	849	10,612	1.57	6.60E-02

Note that significant genes may be contained within multiple enriched terms within a category.
Key: AD, Alzheimer's disease; DAVID, Database for Annotation, Visualization and Integrated Discovery.

synaptic vesicle availability, particularly in the hippocampus. These genes included increased expression of dynamins (DNM1L, DNM3), which are fundamental to synaptic vesicle endocytosis and recycling, along with synaptojanin 1 (SYNJ1) and endophilin1 (SH3GL2), which work in concert and are required for ultrafast endocytosis and clathrin uncoating of endocytosed synaptic vesicles. Several additional anti-aging/AD genes associated with synaptic vesicle endocytosis and recovery were the clathrin adaptor protein complex 2 (AP2M1), the Bloc-1 subunit pallidin (BLOC1S6), syndapin/PACSIN1, amphiphysin (AMPH), secretory carrier membrane protein 5 (SCAMP5), TorsinA (TOR1A), and multiple V-ATPase subunits for ATP6V1 (subunits A, C1, D, E1, G2) (Fig. 3).

Taken together, these data suggest that aging/AD are accompanied by declining expression of multiple components of the synaptic vesicle release, trafficking, and recycling machinery, and that physical activity may counteract these gene expression patterns and maintain a more effective presynaptic machinery.

3.2.2.2. Glutamate and GABA signaling: neurotransmitter levels, receptors, and receptor trafficking. Further analysis of the anti-aging/AD genes showing increased expression with physical activity revealed numerous genes that regulate postsynaptic signaling efficiency of glutamatergic and GABAergic neurotransmission (Supplemental Table 4). These included several genes that regulate glutamate availability (glutaminase [GLS], high-affinity glutamate transporter SLC1A6), glutamate receptor levels (NMDA receptor subunit 2a [GRIN2a], kainate receptor 2 [GRIK2], AMPA receptor subunit 4 [GRIA4]) and auxiliary components that modulate glutamate receptor signaling kinetics (ferric chelate reductase 1 like [FRRS1L], neuropilin and tolloid like 1 and 2 [NETO1, NETO2], homer scaffolding protein 1 [HOMER1]). In addition, there were several genes that regulate glutamate receptor availability in the postsynaptic density (PSD), including genes that promote removal of AMPA GluR1 from the PSD (glutamate receptor interacting protein 1 [GRIP1], ATPase family AAA domain containing 1 [ATAD1], SYNJ1, VAMP2) as well as genes that increase AMPA GluR1 anchoring in the PSD (sortilin-related VPS10 domain containing receptor 3 [SORCS3], leucine-rich repeat transmembrane neuronal 4 [LRRTM4]), GRIN2A clustering in the PSD (rabphilin 3A [RPH3A]) and GRIN2B localization in the PSD (lin-7 homologs A and B [LIN7A,

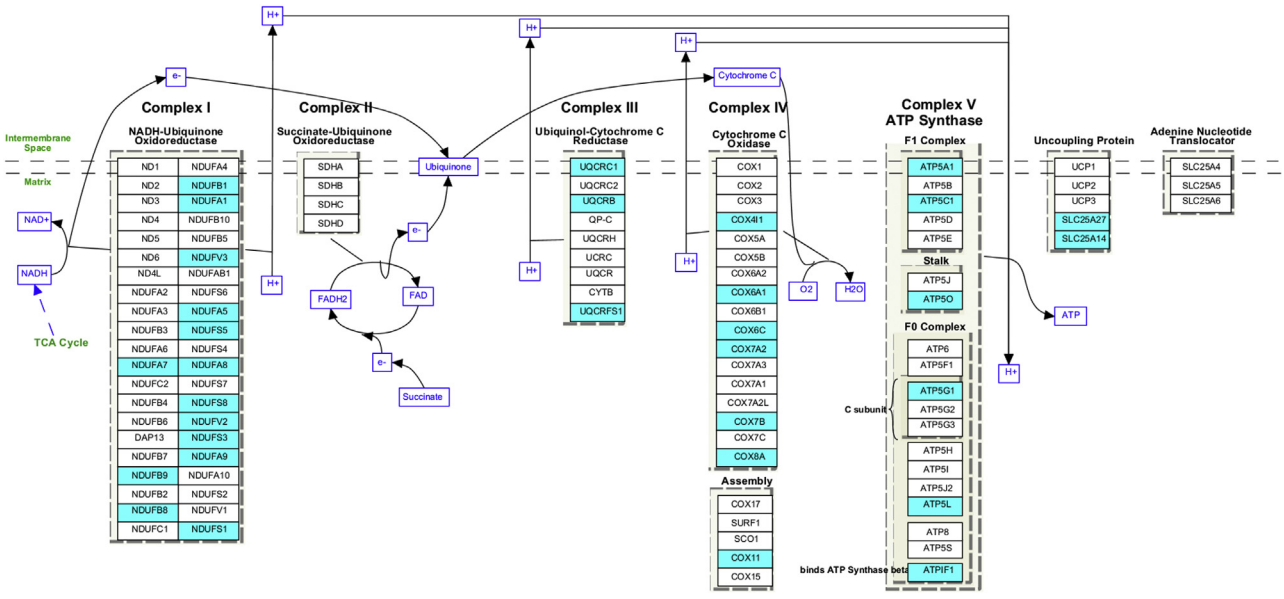

Fig. 2. The KEGG pathway "oxidative phosphorylation" (3.17-fold enriched, $p = 5.5 \times 10^{-7}$) overlaid with microarray data—blue highlight identifies genes showing significantly increased expression in association with physical activity and decreased expression in aging or AD. Significant genes included approximately 30% of the genes encoding mitochondrial complex I (NADH:ubiquinone oxidoreductase) (NDUFA1, NDUFA7, NDUFA8, NDUFA9, NDUFAF5, NDUFB1,NDUFB8, NDUFB9, NDUFS1, NDUFS3, NDUFS5, NDUFV2 and NDUFV3) approximately 35% of the genes encoding complex IV (cytochrome C oxidase) (subunits COX4I1, COX6A1, COX6C, COX7B, COX7A2, COX8A and COX11) and complex V (ATP synthase) (ATP5A1, ATP5C1, ATP5O, ATP5G1, ATP5L, ATP51), as well as 2 components (SLC25A27, SLC25A14) of the uncoupling protein. No genes in the pathway showed decreased expression in association with physical activity. Abbreviations: AD, Alzheimer's disease; KEGG, Kyoto Encyclopedia of Genes and Genomes.

LIN7B]). At the same time, anti-aging/AD genes included genes regulating GABAergic neurotransmission, including increased expression of $GABA_A$ receptor subunits (GABRA1, GABRG2) and gephyrin (GPHN)—the neuronal assembly protein that anchors GABA receptors to the inhibitory PSD—along with several neuromodulators that augment GABA tone (GLRB, SSTR1, cholecystokinin).

These data indicate that physical activity is associated with increased expression of many genes that regulate postsynaptic signaling efficacy of glutamatergic and GABAergic neurotransmission. The constellation of gene expression changes with increased physical activity may function to enhance excitatory signaling and at the same time to dampen responsiveness within neural circuits.

3.2.2.3. Synapse formation, plasticity, and axon health. In parallel, genes showing increased expression in association with physical activity and decreased expression with aging/AD included central organizing molecules which promote new synapse formation and maturation, molecular pathways key for activity-dependent plasticity, and genes supporting axonal function and white matter integrity (Supplemental Table 5).

Notable categories associated with synapse formation, maturation, and plasticity showing enrichment included a 2.2-fold enrichment for genes associated with the "postsynaptic cell membrane" ($p = 7.5 \times 10^{-3}$) and 3.6-fold enrichment for the BioCarta pathway "transcription factor CREB and its extracellular signals" ($p = 6.8 \times 10^{-2}$). Genes regulating spine genesis and synapse maturation included presynaptic neurexins (NRXN1, NRX3) and calsyntenin 3 (CLSTN3)—a synaptogenic adhesion molecule that works in concert with neurexins—along with leucine-rich repeat molecules (LRRTM4, LRRC4C, LRRC7), cell adhesion molecule 2 and 3 (CADM2, CADM3), and other genes: Rho guanine nucleotide exchange factor 7 (ARHGEF7), myocyte enhancer factor 2C (MEF2C), inositol-trisphosphate 3-kinase A (ITPKA), caytaxin (ATCAY), ATPase plasma membrane Ca2+ transporting 2 (ATPB2), phosphatase and actin regulator 1 (PHACTR1), teneurin transmembrane protein 2 (TENM2), diacylglycerol kinase (DGK), glycoprotein M6A (GPM6A), cell division cycle 42 (CDC42), neuralized E3 ubiquitin protein ligase 1 (NEURL1). In addition, several of the anti-aging/AD genes increased in association with physical activity are key molecules that enable dynamic plasticity critical for memory formation. These included kinases that drive the signaling in the nucleus required for sustained long-term potentiation such as the catalytic and regulatory subunits of protein kinase A (PRKACB, PRKAR1A, PRKAR1B, PRKAR2B) and protein kinase C (beta subunit; PRKCB), genes regulating signaling through the CREB transcription factor (e.g., Adenylate cyclase 1 [ADCY1], calcium/calmodulin-dependent protein kinase delta [CAMK2D], GNAS complex [GNAS], growth factor receptor bound [GRB2]), and genes that facilitate trafficking and signaling of brain-derived neurotrophic factor, a key plasticity molecule that facilitates long-term potentiation induction and stability and that is essential for hippocampus-dependent learning (e.g., endophilin [SH3GL2], synaptotagmin 4 [SYT4], ubiquitin specific peptidase 14 [USP14]).

Interestingly, genes showing increased expression in association with physical activity and decreased expression with age/AD were also enriched for genes supporting axonal connectivity and function and white matter integrity. There was strong enrichment for the term "growth cone" (2.4-fold enriched, $p = 1.2 \times 10^{-2}$) (Supplemental Table 6), an actin-supported extension of a developing or regenerating neurite seeking its synaptic target and a 1.8-fold enrichment for "neuron projection" ($p = 9.6 \times 10^{-2}$). It is possible that this gene expression pattern reflects axonal outgrowth from newly generated neurons in the neurogenic subgranular zone of the hippocampus, as increased expression was seen for several genes that regulate axonal outgrowth and targeting

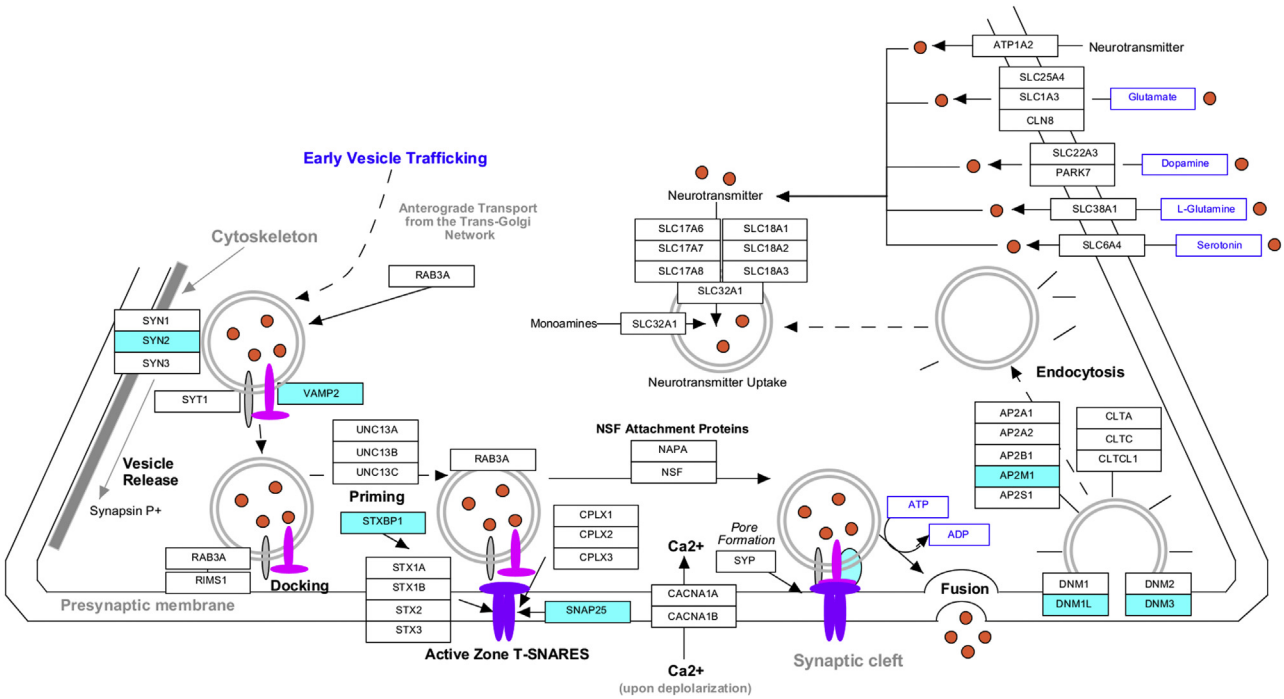

Fig. 3. The synaptic vesicle KEGG pathway overlaid with microarray analysis with blue highlight identifying genes showing significantly increased expression in association with physical activity and decreased expression in aging or AD. Significant genes are involved in synaptic vesicle trafficking, priming, and docking at the active zone: SNAP25, synaptobrevin/VAMP2, synapsin 2 (SYN2), syntaxin binding protein 1 (STXBP1) as well as synaptic vesicle endocytosis and recycling (dynamin-like 1 (DNML1), DNML3, and adaptor protein complex 2 (AP2M1). No genes in the pathway showed decreased expression in association with physical activity. Abbreviations: AD, Alzheimer's disease; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(e.g., RAC1-activated kinases [PAK1, PAK3, PAK5, PAK6], ephrins [EFNB3, EFNA5, and its receptor EPHA3], slit guidance ligand 2 [SLIT2], roundabout guidance receptor 2 [ROBO2], SLIT-ROBO Rho GTPase activating protein [SRGAP3]), along with a number of important genes that enhance proliferation, survival, and migration of immature neurons (Supplemental Table 7) (e.g., doublecortin [DCX], cyclin-dependent kinase 5, glycogen synthase kinase 3 beta [GSK3b], platelet activating factor acetylhydrolase [PAFAH1B1], TMF1-regulated nuclear protein 1 [TRNP1], ephexin 1 [NGEF], ROBO2). However, while stimulation of neurogenesis with physical activity is one of the most consistently documented effects of exercise in the animal hippocampus, there is controversy concerning the capacity for neurogenesis in the adult human hippocampus. It is possible that these expression patterns are not indicative of active neurogenesis but rather reflect promotion of axonal health and function. Indeed, anti-aging/AD genes undergoing decreased expression in the aging/AD hippocampus but increased expression in association with physical activity showed strong enrichment for genes associated with the axon (GOTERM-CC, 2.0-fold enrichment, $p = 3.4 \times 10^{-3}$) (Supplemental Table 8), as well as the myelin sheath (GOTERM-CC, 2.5-fold enriched, $p = 1.0 \times 10^{-4}$) (Supplemental Table 9).

Taken together, these data suggest that late-life physical activity may counteract aging/AD-associated declines in synaptic plasticity by enhancing the molecular machinery for spine formation and activity-dependent plasticity and promoting white matter integrity and axonal connectivity.

3.2.3. Anti-aging/AD genes decreased in association with physical activity are enriched for transcriptional control

DAVID analysis of the 621 anti-aging/AD probesets showing decreased expression with physical activity identified 408 unique and unambiguous DAVID ids of which 378 genes could be mapped to an enrichment term. Anti-aging/AD genes showing decreased expression with physical activity were significantly enriched primarily for transcription-related function (Table 2). Significantly enriched terms notably included "Transcription regulation" (UniProt keyword, 1.5-fold enriched, $p = 8.5 \times 10^{-3}$), "Repressor" (UniProt keyword, 2.0-fold enriched, $p = 1.9 \times 10^{-2}$), "Activator" (UniProt keyword, 1.9-fold enriched, $p = 4.4 \times 10^{-2}$) and "negative regulation of transcription from RNA polymerase II promoter" (GOTERM, 2.1-fold enriched, $p = 8.8 \times 10^{-2}$) (Supplemental Table 10). Transcriptional control genes that were increased with aging/AD but decreased in association with physical activity included, among others, several epigenetic regulators of histones [histone deacetylase 1 (HDAC1), lysine-specific histone demethylase 1B (KDM1B), lysine 9 histone 3 demethylase (KDM3A), histone lysine methyltransferase 2C (KMT2C)], components of the HDAC complex [Sin3A-associated proteins 18 and 30 (SAP18, SAP30), MDS1 and EVI1 Complex Locus Protein MDS1 (MECOM), bHLH Transcription Factor 1 (TAL1), Transformer-2 Alpha (TRA2A), numerous zinc finger proteins (ZFHX3, ZNF24, ZNF37A, ZNF160, ZNF274, ZNF397, ZNF532, ZNF652, ZN721, ZBTB20, ZBTB47), along with the transcription factor SP3, which recruits HDAC2 to suppress transcription of synaptic plasticity–associated genes. Importantly, these genes are ones that show increased expression with aging and/or AD but decreased expression in association with physical activity, suggesting that physical activity may offset transcriptional dysregulation and help maintain appropriate transcriptional control of gene expression in the aging and AD hippocampus.

3.3. Replication: Nanostring technology

NanoString nCounter gene expression system was used to assess expression levels of an expanded set of human hippocampal tissues that included 12 additional cases to those used in the microarray analysis. Gene expression was compared in low (n = 18) versus high activity (n = 16) cases for 13 synaptic genes that showed decreased expression with aging/AD and significantly increased expression with physical activity (GABRG2, GLRB, GRIN2a, SLC1a6, SYN2, VAMP2, SNAP25, USP14, NRXN1, NRXN3, SYT4, SSTR1, Munc13-3) and for 10 genes where microarray analysis demonstrated significantly decreased expression with aging/AD but only trends ($p < 0.1$) for increased expression with physical activity (GABBR1, GABRA2, GABRA4, GABRB3, GABRG1, GRIA2, DOC2a, SCN2b, DLG3/SAP102, AKAP5).

Nanostring analysis using an expanded set of samples demonstrated increased gene expression with high physical activity for GABRG2 ($p = 0.050$), GRIN2a ($p = 0.036$), GLRB ($p < 0.033$), SLC1a6 ($p = 0.019$), SYN2 ($p = 0.014$), USP14 ($p = 0.009$), NRXN1 ($p = 0.008$), SYT4 ($p = 0.027$), SSTR1 ($p = 0.029$), and UNC13c/Munc13-3 ($p = 0.049$), confirming the microarray data, whereas expression for NRXN3 ($p = 0.065$), VAMP2 ($p = 0.096$) and SNAP25 ($p > 0.10$) failed to reach statistical significance. The nanostring analysis additionally demonstrated increased gene expression with high physical activity for GABBR1 ($p = 0.003$), GABRA2 ($p = 0.002$), GABRA4 ($p = 0.017$), GABRB3 ($p = 0.011$), GABRG1 ($p = 0.0006$), GRIA2 ($p = 0.050$), DOC2a ($p = 0.008$), DLG3/SAP102 ($p = 0.038$), and AKAP5 ($p = 0.021$) and a trend for SCN2b ($p = 0.065$) (Fig. 4).

Finally, we analyzed the NanoString data using only the subset of NanoString cases that represented the 12 new cases not used in the microarray (n = 8 low activity, n = 4 high activity) as an independent confirmation of the microarray data. 100% of the genes showed directional agreement between the NanoString and microarray data. Mann-Whitney comparison of average values across the genes revealed significantly higher expression in the high-activity group versus low-activity group [medians: 0.894 (low), 1.12 (high); $p < 0.0001$]. The data thus confirm in an independent set of experimental samples that expression levels in the nanostring-measured genes, as a group, moved in the same direction as in the microarray analysis.

4. Discussion

Multiple studies have established that physical activity is associated with delayed age-related decline and onset of AD in humans (Best et al., 2017; Buchman et al., 2012; Erickson et al., 2012; Hillman et al., 2008; Kirk-Sanchez and McGough, 2014; Korol et al., 2013; Prakash et al., 2015; Suwabe et al., 2018; Weuve et al., 2004; Yaffe et al., 2001). This study sought to identify underlying mechanisms by which physical activity supports brain health and slows aging/AD-related declines in hippocampal function. Using microarrays, hippocampal transcription patterns associated with late-life physical activity levels were compared with hippocampal gene expression changes associated with aging and AD. The data demonstrate that late-life physical activity is associated with major reprogramming of hippocampal gene expression, with a striking number of these genes showing significant but opposite patterns of expression change in aging/AD. The majority (>70%) of these anti-aging/AD genes show increased expression in association with physical activity but decreased expression in aging/AD, with an extensive representation of genes regulating mitochondrial energy production, synaptic plasticity at the structural and signaling levels, and axon function.

With age, a decline in energy production has been reported across organs and multiple species (Lane et al., 2015; Lin and Beal, 2006). Our data show that physical activity is linked to increased gene expression that included multiple components of the electron transport chain along with key genes driving the TCA cycle, which operates in the mitochondrial matrix and provides key

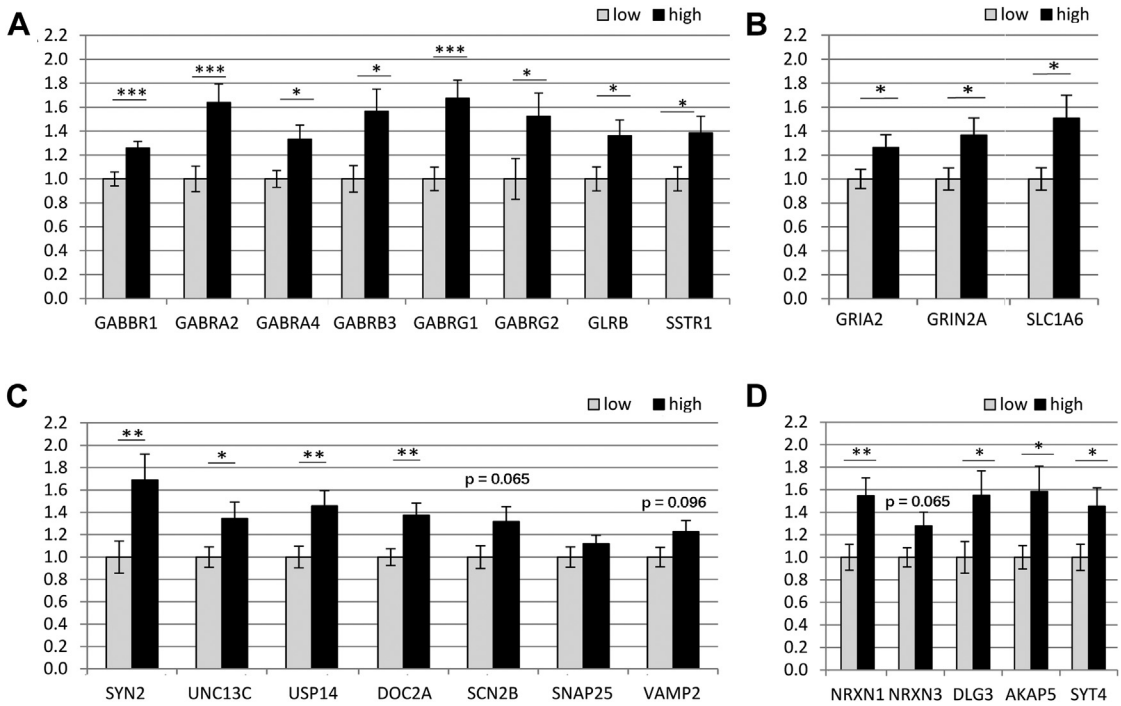

Fig. 4. The NanoString nCounter gene expression system was used to assess gene expression changes with physical activity, using an expanded set of human hippocampal samples (low activity, n = 18; high activity, n = 16). (A) Eight genes associated with GABA signaling: the G-protein–coupled metabotropic $GABA_B$ receptor 1 (GABBR1), ionotropic $GABA_A$ receptor alpha subunits (GABRA2, GABRA4), $GABA_A$ receptor beta subunit 3 (GABRB3), $GABA_A$ receptor gamma subunits (GABRG1, GABRG2), glycine receptor beta (GLRB), and somatostatin receptor 1 (SSTR1). (B) Three genes regulating glutamate signaling: glutamate ionotropic receptor AMPA type subunit 2 (GRIA2), glutamate ionotropic receptor NMDA type subunit 2A (GRIN2a), and the neuron-specific sodium-dependent glutamate/aspartate transporter (solute carrier family 1 member 6: SLC1a6). (C) Seven genes involved in synaptic vesicle trafficking and release: Synapsin II (SYN2), Unc-13 Homolog C (UNC13c/Munc13-3), ubiquitin-specific peptidase 14 (USP14), double C2 domain alpha (DOC2a), voltage-gated sodium channel (type II, subunit B: SCN2B), synaptosomal-associated protein 25 (SNAP25), and vesicle-associated membrane protein 2 (VAMP2). (D) Five genes involved in building new synapses and the maintenance and plasticity of the PSD: neurexin 1 (NRXN1), neurexin 3 (NRXN3), discs large homolog 3 (DLG3, also known as synapse-associated protein SAP102), A-kinase anchoring protein 5 (AKAP5), and synaptotagmin 4 (SYT4). mean ± SEM. T-tests (excel) * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.005$.

intermediaries (NADH and FADH2) to the electron transport chain and enhances energy production. Importantly, the genes showing increased expression with physical activity are the same genes that undergo reduced expression in aging and AD. Enhancement of mitochondrial function may serve as a core mechanism by which physical activity delays aging/AD-related cognitive decline, considering that a ready supply of energy is essential for the maintenance of synaptic fidelity (Harris et al., 2012; Hebert-Chatelain et al., 2016; Smith et al., 2016).

One of the major causes of cognitive decline in aging and AD is synaptic dysfunction and ultimately failure that progressively disrupts connectivity patterns critical to ongoing functions of the brain (Koffie et al., 2011; Prieto et al., 2017; Selkoe, 2002). Our data suggest that gene expression patterns associated with physical activity may promote synaptic fidelity in the aged hippocampus not only by targeting energy production but also by counteracting aging/AD-related declines in synaptic components and processes central to neurotransmission. Notably, anti-aging/AD genes showing increased expression in association with physical activity included many presynaptic components that regulate synaptic vesicle priming, release and recycling, postsynaptic GABA and glutamate neurotransmitter receptors that control the excitatory/inhibitory balance, and PSD scaffolding molecules that tune NMDA and AMPA glutamate signaling, all of which are major mechanisms to regulate synaptic efficacy. In addition, many of the anti-aging/AD genes showing increased expression with physical activity target myelin and axon health, suggesting that physical activity may promote white matter integrity and axonal connectivity and function. The strong relationship between physical activity and expression of genes targeting axon functioning are important, given that white matter degeneration and reduced axonal efficacy are notable components of cognitive decline in both normal aging and in AD pathogenesis (Bartzokis et al., 2003; Fletcher et al., 2018; Kruggel et al., 2017).

Approximately 30% of the anti-aging/AD genes showed decreased expression with physical activity but increased expression in aging/AD. Interestingly, these genes were primarily enriched for transcriptional regulation, including many genes driving negative regulation of transcription. Our data suggest that aging/AD is accompanied by augmented transcriptional suppression and that physical activity may offset transcriptional dysregulation and help maintain appropriate transcriptional control of gene expression in the aging and AD hippocampus. The transcriptional control genes that were increased with aging/AD but decreased in association with physical activity included several epigenetic regulators of histones including lysine-specific histone demethylases (KDM1B, KDM3A), histone lysine methyltransferase (KMT2C), histone deacetylase (HDAC1), and components of the HDAC complex (SAP18, SAP30, MECOM, TAL1), along with multiple transcriptional repressors and activators. The possibility that physical activity may reduce aging/AD-associated transcriptional dysregulation would represent a powerful mechanism that could have far-reaching consequences for hippocampal function. For example, one gene of interest is the transcription factor SP3, which forms a chromatin-modifying complex with HDAC2 that suppresses transcription of synaptic-plasticity–associated genes, by removing acetylation from the promoter regions (Yamakawa et al., 2017). Previous studies demonstrate that SP3 is elevated in brains of patients with AD and

in mouse models of AD, and that reducing SP3, or the SP3-HDAC2 interaction, improves synaptic plasticity and cognitive function without affecting HDAC2 function in other processes (Yamakawa et al., 2017). Our data reveal that SP3 is significantly elevated in the hippocampus already with aging, shows a further increase with AD, and is reduced in association with physical activity, which may be one mechanism by which physical activity can facilitate synaptic gene expression in the hippocampus. Consistent with this idea, our data reveal that several synaptic target genes that are negatively co-regulated by HDAC2 and SP3 (Yamakawa et al., 2017) show increased expression in association with physical activity, including GRIK2, LIN7A, SYNGR3, and DLGAP1. Taken together, these data suggest that physical activity may offset aging/AD-associated transcriptional dysregulation and help maintain appropriate gene expression in the hippocampus.

Although many aspects of human brain aging appear to be counteracted by transcription patterns associated with physical activity, an unexpected finding was that physical activity was not associated with extensive involvement of genes regulating immune function and inflammation. We had hypothesized that physical activity would be associated with decreased expression of this gene class based on the literature that the hippocampus undergoes extensive immune gene activation in aging and AD (Barrientos et al., 2015; Berchtold et al., 2008; Cribbs et al., 2012) and the literature that physical activity can constrain activation of immune/inflammatory processes in the hippocampus of animal models (Barrientos et al., 2011; Kohman et al., 2013; Littlefield et al., 2015). Although analysis of the anti-aging/AD genes showing decreased expression in association with physical activity revealed some over-representation of immune/inflammation-related genes, the enrichment did not meet statistical significance. Nonetheless, we observed decreased expression in association with physical activity of a few notable genes that drive immune/inflammatory responses, including several activators of the NF-kB pathway, such as adipocyte enhancer-binding protein 1 (AEBP1), CD40 and filamin A (FLNA), all of which have been implicated in the progression of AD pathology (Giunta et al., 2010; Shijo et al., 2018; Wang et al., 2012). Overall, however, our data did not reveal a strong relationship between physical activity levels and expression of immune/inflammation-related genes, particularly for immune/inflammation-related genes that show increased expression with aging and AD.

The transcriptional signatures identified in our study are supported by the human and animal literature reporting widespread benefits of lifestyle activity to brain structure and function. Imaging studies in humans have demonstrated that exercise increases hippocampal volume and density along with cortical gray and white matter volumes and integrity, notably in brain regions that undergo aging-associated atrophy and functional decline (Colcombe et al., 2006; Erickson et al., 2011; Kleemeyer et al., 2016; Ruscheweyh et al., 2011; Voss et al., 2013). Such changes are consistent with our transcriptional data suggesting that physical activity promotes synaptic and cellular health from augmented energy availability, synaptic function, and axon integrity. The findings in human studies are paralleled by the animal literature demonstrating structural and functional changes in the hippocampus and cortex with physical activity including synaptogenesis and increased dendritic complexity (Eadie et al., 2005; Stranahan et al., 2007), hippocampal neurogenesis (Erickson et al., 2013; Fabel et al., 2009; Gould et al., 1999; van Praag et al., 1999b), enhanced synaptic plasticity (Farmer et al., 2004; van Praag et al., 1999a), improved myelin integrity (Zhang et al., 2017; Zhou et al., 2018), and improved spatial learning and memory (Berchtold et al., 2010; Creer et al., 2010; Fordyce and Wehner, 1993; Intlekofer et al., 2013; van Praag et al., 2005). In addition, animal studies have demonstrated that exercise targets multiple aspects of mitochondrial function (Cechella et al., 2017; Kim et al., 2010; Lee et al., 2014; Marques-Aleixo et al., 2012; Rampon et al., 2000; Stranahan et al., 2008; Tong et al., 2001). Finally, a handful of animal studies have investigated anti-aging/AD benefits of physical activity, demonstrated that exercise reverses age or AD-related changes in hippocampal gene expression, most notably for genes associated with mitochondrial function, synaptic plasticity, and axon/myelin integrity (Boveris and Navarro, 2008; Choi et al., 2018; Kohman et al., 2011). We note that some of the findings of animal studies were not recapitulated in the human brain (e.g., immune gene responses). The discrepancy between our data and the animal literature may be a function of differences in the extent or intensity of exercise participation between humans in this study versus animals, with the exercise undertaken in animal studies generally being of higher frequency (daily), intensity, and distance (>2 km) but usually shorter duration (<4 weeks).

Taken together, our results suggest that physical activity may broadly shift multiple biological processes in the aged hippocampus toward improved functioning; stimulating core functions that are critical for maintaining brain health and synaptic function but that undergo aging/AD-related decline. We note that most of these genes followed a pattern of only modest expression change in the course of cognitively normal aging followed by progressively greater expression change in AD, and conversely, small expression change with moderate physical activity followed by a relatively large expression change in the highest tier of physical activity. Although a relatively modest expression change of any individual gene on its own may not significantly impact hippocampal plasticity and cognitive function, the cumulative effect of reduced expression of multiple genes in a particular functional category, and the gestalt effect of compromised function across multiple categories (e.g., energy production, synaptic efficacy, axon function, transcriptional control), may well be a root cause of age-related decline in hippocampal plasticity and cognitive function. Similarly, the benefit of physical activity to delay age-related cognitive decline and onset of AD may well arise from the cumulative expression change of multiple genes across multiple categories of function. By preserving a more youthful profile across multiple biological processes, these transcriptional effects could empower the brain to strengthen plasticity and build cognitive reserve, providing several potential mechanisms for the cognitive-preserving effects of lifestyle. Although the data presented here are associational and do not provide direct evidence of a causal relationship, these gene expression patterns may guide the combinatorial use of pharmaceutical agents that target biological domains untouched or minimally associated with physical activity in the human hippocampus transcription pattern.

Disclosure

The authors declare no biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2019.02.012.

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