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Differential proteomic and behavioral effects of long-term voluntary exercise in wild-type and APP-overexpressing transgenics

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Abstract

Physical exercise may provide protection against the cognitive decline and neuropathology associated with Alzheimer's disease, although the mechanisms are not clear. In the present study, APP/PSEN1 double-transgenic and wild-type mice were allowed unlimited voluntary exercise for 7 months. Consistent with previous reports, wheel-running improved cognition in the double-transgenic mice. Interestingly, the average daily distance run was strongly correlated with spatial memory in the water maze in wild-type mice ($r^2 = .959$), but uncorrelated in transgenics ($r^2 = .013$). Proteomics analysis showed that sedentary transgenic mice differed significantly from sedentary wild-types with respect to proteins involved in synaptic transmission, cytoskeletal regulation, and neurogenesis. When given an opportunity to exercise, the transgenics' deficiencies in cytoskeletal regulation and neurogenesis largely normalized, but abnormal synaptic proteins did not change. In contrast, exercise enhanced proteins associated with cytoskeletal regulation, oxidative phosphorylation, and synaptic transmission in wild-type mice. Soluble and insoluble A β 40 and A β 42 levels were significantly decreased in both cortex and hippocampus of active transgenics, suggesting that this may have played a role in the cognitive improvement in APP/PSEN1 mice. β -secretase was significantly reduced in active APP/PSEN1 mice compared to

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sedentary controls, suggesting a mechanism for reduced A β . Taken together, these data illustrate that exercise improves memory in wild-type and APP-overexpressing mice in fundamentally different ways.

Introduction

Alzheimer's disease is the most common form of dementia, characterized by plaques of misfolded amyloid β (A β), neurofibrillary tangles of hyperphosphorylated tau, widespread neurodegeneration, and loss of cognitive and other functions. Considerable evidence now supports the notion that factors affecting cardiovascular function may also affect the symptoms and severity of Alzheimer's disease. Indeed, obesity, glucose intolerance, and high cholesterol are all considered risk factors for Alzheimer's disease. Inversely, evidence suggests that physical exercise may improve memory in Alzheimer's patients and perhaps slow cognitive decline. Taken together, these results suggest that metabolic factors may play a significant role in Alzheimer's dementia.

Like Alzheimer patients, transgenic mice that overexpress mutant human amyloid precursor protein (APP) typically show improved memory when given the opportunity to exercise. The improved cognition is usually accompanied by reductions in A β and amyloid plaques. However, improvements in memory and pathology are not always observed, and there is little understanding of how exercise-induced changes may come about. Adlard et al. (2005) observed a substantive reduction in plaque load and total A β in 6-month-old TgCRND8 mice given the opportunity for voluntary wheel-running for 5 months. However, they did not observe changes in APP, α -, β -, or γ -secretase activity, APP C-terminal fragments (CTFs), or degradative enzymes. They repeated the study in a separate group of mice given access to running wheels for only 1 month, starting at 6 weeks of age. Again, A β was reduced in the presence of normal levels of APP, secretase activity, insulin degrading enzyme (IDE), and neprilysin. However, this time α - and β -CTFs were significantly reduced. They concluded that transient changes in APP processing occurred early in the exercise routine, resulting in long-lasting reductions in A β . In contrast, Maesako et al. (2012) observed reduced CTFs after 5 months of voluntary exercise in 7.5-month old J20 mice. Although the J20 and TgCRND8 transgenic lines harbor the same Swedish (K670N/M671L) and Indiana (V717F) mutations, they are driven by different promoters and the mice are on different background strains. These and other methodological differences may produce large differences in outcomes. Indeed, voluntary running, forced running (e.g., shock-motivated), and swimming have all been used to induce activity-related changes in APP-overexpressing transgenics. Exercise can also improve memory in wild-type mice in the absence of mutant APP or A β , so non-amyloidogenic factors may be partly responsible for, or interact with, the reductions in A β to improve memory. Voluntary exercise in wild-type mice has been associated with increases in neurogenesis, neurotrophic factors, synaptogenesis, and gluconeogenesis. Thus, despite the numerous papers addressing the effects of physical activity in Alzheimer models, we do not have a clear understanding of how exercise clears A β pathology and improves memory. In the present study we used a novel 24-h home-cage running wheel system that eliminates non-running artifacts and avoids potential nuisance factors such as aversive

control or exposure to a running wheel for a short period every day. Control mice were treated identically except that running wheels were inoperable.

Methods

Subjects

The APP/PSEN1 double-transgenic mice (#004462, Jackson Laboratories, Bar Harbor, ME) incorporate a chimeric human/murine amyloid precursor protein (*APP*) construct bearing the Swedish double mutation (K595N/M596L) and the exon-9-deleted human presenilin 1 (*PSEN1*) mutation (APPSwe+PSEN1/ E9), both controlled by the mouse prion protein promoter (PrP) to drive expression mainly in CNS neurons. The transgenics are maintained as double-hemizygotes by periodic backcrossing to wild-types of the B6C3F1 strain (#100010, Jackson Laboratories). These double-transgenic mice have spatial memory deficits as early as 7 months of age (Bernardo et al., 2009; Reiserer et al., 2007). Mice were group-housed in standard tub cages with 3–5 per cage, until 5 months of age, at which time they were individually housed in similar caging, each outfitted with a running wheel (9–12 per group). Hippocampal and cortical tissue from the same mice were used for proteomics (7–10 per group), ELISA (7–8 per group) and Western (2–8 per group) assays. Mice were sacrificed 24 hours following water-maze probe trial.

Exercise

Our computer interfaced, automated exercise monitoring system collected data continually for 7 months, except for ~1 min. each week needed to replace the dirty cage. This system incorporates a commercially available running wheel (Fast-Trac #K3251, Bio-Serv, Frenchtown, NJ) and uses standard mouse housing cages. Half of the mice of each genotype had free access to functional running wheels (active groups), while the other half had access to otherwise identical non-functional running wheels (inactive groups) to control for potential environmental enrichment from presence of the running wheel. The running-wheel system uses infrared emitters and detectors to sense wheel motion (Fig. 1), and provides high-resolution data that can be used to study not only speed and duration of running, but multiple levels of structure and rhythms, and running direction preferences (clockwise vs. counter-clockwise). Data were collected through tracking of nine equally-spaced reflective strips attached to the outside of the running wheels, each of which provided an infra-red “pulse” input. Unlike other running wheel systems, our design eliminated false signals generated by wheel rocking and continuous sensor-detector proximity through fail-safes programmed into the software. Each running-wheel input generated a separate indexed data file which was imported into a database or spreadsheet program for analysis. The time elapsed between pulses for a single sensor provided speed data, while the order of leading edges between sensors provided direction data. The signals were processed by a LabView (National Instruments, Austin, TX) program that counted one revolution for every nine pulses in the same direction.

Memory

Spatial cognition was assessed using a standard reference-memory task the water maze as previously described (Bernardo et al., 2009; Dhanushkodi and McDonald, 2011). Each

mouse received four trials per day in a 92-cm diameter pool with a clear acrylic platform (10-cm diam.) submerged 0.5 cm below the surface of the water. Digital images were captured and analyzed using macros especially written for the water maze, run in the public-domain software NIH Image (Bazalakova et al., 2007; Miyakawa et al., 2001). Trials were conducted in a spaced fashion, i.e., each subject received its first trial before the first subject received its second trial. A 60-s probe trial was conducted without the platform to assess memory for the former platform location, 24 hours following the final acquisition session. The amount of time spent in a 30-cm-diam. zone surrounding the former platform location was used as a measure of memory during the probe trial. This zone score was used as a more accurate measure of memory, compared to quadrant time (Bernardo et al., 2007; Dhanushkodi and McDonald, 2011; Flanigan et al., 2014; Harrison et al., 2009a). Because of the shape of the pool quadrants, some locations within the target quadrant are farther away from the platform location than other locations in neighboring quadrants. For example, in a 92-cm-diam. pool a mouse might be as far as 32 cm from the platform but still inside the target quadrant, and as near as 17 cm from the platform but in a neighboring quadrant. Comparing time spent in the 30-cm zone to equivalent zones in the other three quadrants is a more precise measure of memory because it eliminates the problems caused by the shape of the quadrant, and excludes adventitious swimming in the 8-cm periphery (Fig. 2b). We did not conduct a cued-platform control task for several reasons. First, we and others have already shown that the APP/PSEN1 mice have robust spatial learning impairments in the water maze but are unimpaired on the non-spatial cued control task (Bernardo et al., 2007; Cao et al., 2007; Cohen et al., 2009; Harrison et al., 2009b; Harrison et al., 2010; Lewis et al., 2010). Second, the transgenics also exhibit deficits in cognitive flexibility (Arrazola et al., 2009; Reiserer et al., 2007; Toledo and Inestrosa, 2010), a putatively frontally-mediated cognitive process involved in, e.g., switching learning modalities from cued to spatial. Because our focus was primarily on hippocampal pathology and proteins, we wanted the water-maze task to reflect pure spatial learning deficits as much as possible. Although we could have conducted the cued control condition following the spatial version, we wanted the neurochemistry to reflect as much as possible the changes associated with spatial learning. To do this, we had to sacrifice the mice as soon as possible after the probe trial. For these reasons, we omitted the cued-platform phase in the present study.

Neurochemistry

Mice were sacrificed at 12 months of age. Brains were dissected rapidly on wet ice and bilateral hippocampi and overlying cortex were flash-frozen. Tissue was homogenized and proteins extracted from each sample. Soluble and insoluble A β 40 and A β 42 were measured by ELISA (#27718 & 27712, Immuno-Biological Laboratories, Minneapolis, MN) as previously described (Bernardo et al., 2009). Western blot for full-length amyloid precursor protein (APP) was conducted using 1E6 and LN27 antibodies as previously described (Bernardo et al., 2009). Westerns were also conducted for β -amyloid converting enzyme 1 (BACE1; #SAB2104731, Sigma-Aldrich, St. Louis, MO), sAPP α (gift of Dr. Fang Liao of UTHSC), protein kinase C γ (PKC γ ; # SAB2104731, Sigma-Aldrich).

Proteomics sample preparation

Protein extraction was carried out by homogenizing the tissue in buffer consisting of proteinase and phosphatase buffers using Tissue Tearor (Biospec Products, Bartlesville, OK). Protein concentrations were determined with the Pierce BCA protein assay (Thermo Fisher Scientific, Waltham, MA). SDS sample buffer was added directly to the protein extracts, boiled and resolved in a short stack (5 min) by 4–12% SDS Bis-Tris PAGE (Invitrogen, Carlsbad, CA), stained overnight with colloidal blue (Invitrogen), then destained with ddH₂O for 4 h. The entire stained region was excised, equilibrated in 100 mM ammonium bicarbonate, reduced, carbamidomethylated, further destained, dehydrated, and finally digested with Trypsin Gold according to the manufacturer's protocol (Promega, Madison, WI). Following digestion, peptides were concentrated under vacuum and resolubilized in 0.1% formic acid prior to 1D reverse phase nLC-ESI-MS analysis.

Peptide analysis by liquid chromatography-electrospray ionization-tandem/mass spectrometry (LC-ESI-MS/MS)

For each sample, 1 µg of peptide digest was analyzed using an LTQ XL ion-trap mass spectrometer equipped with a nano-electrospray source and a Surveyor plus binary high-pressure liquid chromatography (HPLC) pump (Thermo Scientific, San Jose, CA) using a split-flow configuration. Separations were carried out using a 150 µm × 13 cm pulled-tip Jupiter 5-µm 300-Å C18 column (Phenomenex, Torrance, CA). The HPLC was set up with two mobile phases that included solvent A (0.1% formic acid in ddH₂O) and solvent B (0.1% formic acid in 85% ddH₂O/15% acetonitrile), and was programmed as follows: 0% of solvent B for 15 min (2 µL/min, load), 0%–50% of solvent B for 65 min (~0.5 nL/min, analyze), and 0% of solvent B for 20 min (2 µL/min, equilibrate). The LTQ XL was operated in data-dependent triple-play mode, with a survey-scan range of 300–1200 *m/z*, followed by a zoom scan for charge-state determination for each MS², which were carried out with 2.0 dalton isolation widths on the three most intense ions. MS data were collected in profile mode for all scan types. Charge-state screening and dynamic exclusion were enabled with a minimal signal intensity of 2000, a repeat count of 2, and an exclusion duration of 90 s for ions ± 1.5 *m/z* of the parent ion. The automatic gain control settings were 3.0×10^4 and 5.0×10^3 ions for survey and zoom modes, respectively. Scan times were set at 25 msec and 50 msec for survey and zoom modes, respectively. For collision-induced dissociation, the activation time, activation Q, and normalized collision energy were set at 10 msec, 0.25, and 35%, respectively. The spray voltage was set at 1.9 kV with a capillary temperature of 170° C. All runs were carried out in duplicate from the same samples.

Protein identification and quality control

The XCalibur RAW output files were centroided, converted to MzXML format, and the resulting mgf files were then created using both ReAdW and MzXML2Search respectively (<http://sourceforge.net/projects/sashimi/>). The data were searched using SEQUEST (v.27 rev12, .dta files), set for two missed cleavages, a precursor mass window of 0.45 Da, tryptic enzyme, variable modification M @ 15.9949, and static modifications C @ 57.0293. Searches were performed with a mouse subset of the UniRef100 database, which included

common contaminants such as digestion enzymes and human keratins. Identified peptides were filtered, grouped, and quantified using ProteoIQ (Premierbiosoft, Palo Alto, CA). Only peptides with charge state of $\geq 2+$, a minimum peptide length of 6 amino acids were accepted for analysis. ProteoIQ incorporates the two most common methods for statistical validation of large proteome datasets, false discovery rate (FDR), and protein probability (Keller et al., 2002; Nesvizhskii et al., 2003; Weatherly et al., 2005). The false discovery rate (FDR) was set at $< 1\%$ cut-off, with a peptide probability of ≥ 0.5 , a protein group probability of ≥ 0.7 , and number of unique peptides per protein of ≥ 2 . Relative quantification was performed via spectral counting, and spectral count abundances were normalized between samples.

Statistical analysis

Most behavioral, histological, and neurochemical data were analyzed using one-and two-way factorial analyses of variance (ANOVAs), followed when appropriate with orthogonal paired comparisons. Tukey's HSD was used for non-orthogonal *post-hoc* comparisons to restrict familywise error. To correlate neurochemical with behavioral data, a composite cognition score was computed from the mean of the last four days of water-maze acquisition, when mice were performing asymptotically, and the time spent in the 30-cm target zone during the probe trial. To calculate the composite measure, the inverse of the acquisition score was taken so that "good" cognition for each measure was a higher number. Each mouse's score was then converted to a z score based on the mean and standard deviation of all mice for the particular measure. Finally, the mean of the two z scores was taken as the composite cognition score, with a population mean of 0.0 and a standard deviation (SD) of 1.0. The mean distance (km) run per day for the last 30 days was used as the activity score for each mouse. Time-series data were analyzed using hierarchical linear modeling, with time as a continuous repeated measure and subject as a nominal random factor nested within exercise group and genotype.

Filtered data for 1460 proteins were first analyzed using 2×2 factorial ANOVAs, with genotype and running-wheel status as between-subjects factors. Paired follow-up comparisons were conducted using Fisher's protected LSD test on those proteins having significant main or interaction effects on the omnibus ANOVA. To avoid spurious Type I errors, follow-up comparisons were restricted to *a priori* hypotheses of interest, i.e., inactive wild-type vs. inactive APP/PSEN1, active wild-type vs. inactive wild-type, and active APP/PSEN1 vs. inactive APP/PSEN1. Initial ELISA data were analyzed using multivariate ANOVAs (MANOVAs) fitted to identity matrices. Pearson product-moment correlations were conducted among running, cognition, and A β measures. All statistical tests were two-tailed with α set at .05.

Results

Mice ran for 7 months on the running wheels, and data were collected every day. There were no significant differences in the distance run per month [Fig. 2a; genotype $F(17) < 0.1$, $p = .934$; Genotype \times Month $F(1,107) < 0.1$, $p = .875$]. The time run per day and speed of running also did not differ by genotype [F 's < 1.6 , p 's $> .227$], and were both significantly

correlated with distance [r 's $> .595$, p 's $< .012$]. Consistent with our previous studies showing spatial learning deficits in these transgenics, the inactive APP/PSEN1 mice performed significantly worse than their inactive wild-type counterparts during water-maze acquisition [Fig. 2c, $F(1,36) = 10.8$, $p = .0022$; Genotype \times Session $F(1,316) = 2.3$, $p = .0301$]. There was also a significant main effect for running-wheel status, demonstrating that mice with active running wheels performed better in the water maze than the sedentary groups [$F(1,36) = 13.3$, $p = .0008$]. Genotype \times Activity interactions were not significant (F 's < 2.7 ; p 's $> .112$). Follow-up analyses showed that exercise significantly improved learning only in the transgenic mice [$F(1,20) = 11.0$, $p = .0035$]. Although enhancement of learning in wild-type mice was substantive, inactive wild-type controls were already performing proficiently and thus exercise-induced improvement was not statistically significant [$F(1,16) = 3.9$, $p = .067$]. Swim speed did not differ significantly by genotype [F 's < 0.5 ; p 's $> .51$] or running-wheel status [F 's < 4.1 ; p 's $> .052$].

On the probe trial, the main effects for genotype and running-wheel status were not statistically significant with respect to time spent in the 30-cm target zone compared to the mean time in equivalent zones in the other three quadrants [Fig. 2e; F 's < 4.1 ; p 's $> .052$]. However, there was a significant Zone \times Activity interaction [$F(1,34) = 4.7$, $p = .0364$]. Follow-up tests by group showed that the inactive wild-type mice spent significantly more time in the target zone than the mean of the other three zones ($p = .0323$). This was not the case for the inactive APP/PSEN1 mice ($p = .144$), consistent with the memory deficits previously demonstrated in these mice (Bernardo et al., 2009; Reiserer et al., 2007). When given the opportunity for exercise, both wild-type and APP/PSEN1 groups showed selective search for the target zone (p 's $< .003$). On the composite cognition score, there were significant main effects for genotype [$F(1,34) = 8.1$, $p = .0075$] and running-wheel status [Fig. 2d; $F(1,34) = 10.1$, $p = .0031$]; the interaction effect was not significant [$F(1,34) = 0.4$, $p = .554$]. To determine whether the improvement in memory was related to the extent that mice ran, we conducted correlations between the composite cognition score and the average distance per day during the last month on the running wheels. Figure 2f shows that cognition correlated tightly with the amount of exercise in wild-type mice [$r(7) = .9791$, $p < .0001$] but not in transgenics [$r(6) = -.114$, $p = .770$].

Factorial analyses of filtered protein data revealed that 194 proteins differed significantly with respect to genotype, running-wheel status, and/or the interaction between the two. Follow-up analyses on these protein showed that 42 proteins differed between the inactive wild-type and APP/PSEN1 groups; 30 proteins differed significantly between the wild-type active and inactive groups; and 27 proteins differed between the APP/PSEN1 active and inactive groups. Notably, only two of the 30 proteins that changed in response to running-wheel activity in the wild-type mice also changed in the active transgenics. Initial follow-up analyses were conducted to determine baseline differences between wild-type and transgenic animals with non-functional running wheels, i.e., to determine which changes are specifically relevant to APP overexpression and A β -associated neuropathology in the absence of wheel running. Of the 42 proteins that differed between the two inactive groups, 30 were overexpressed and 12 were reduced (Table 1). Most of the proteins expressed differentially between wild-type and APP/PSEN1 inactive groups clustered into pathways involved in synaptic transmission, cytoskeletal regulation, neurogenesis, oxidative

phosphorylation, protein processing, and apoptosis. Three proteins differed with respect to glycolysis/ gluconeogenesis, all involved in pyruvate metabolism. Glial fibrillary acidic protein (GFAP) and heat shock 90- α (HSP90- α) were also significantly changed in APP/PSEN1 mice, indicative of the elevated neuroinflammation previously reported in these transgenics (Bernardo et al., 2009). Most prominent among the significantly-different proteins were a 20-fold increase in voltage-dependent anion channel 1 (VDAC1), an 18.6-fold increase in protein kinase A regulatory subunit 2 β (PKA2 β), and a 10-fold increase in protein kinase C γ (PKC γ). These proteins are involved in synaptic transmission, mitochondrial dysfunction, and apoptosis, and together suggest dysregulation of calcium signaling in the APP/PSEN1 mice. We validated these proteins using Western blot to confirm the proteomics results. Figure 3a shows that among sedentary mice, all three of the proteins were more highly expressed in APP/PSEN1 transgenics. The Genotype \times Exercise interaction was significant for PKC γ [$F(1,14) = 4.9, p = .0437$], as was the main effect for genotype [$F(1,14) = 5.8, p = .0310$]. The main effect for running-wheel status was not significant [$F(1,14) = 3.9, p = .069$]. Tukey follow-up test showed that the inactive wild-type mice had significantly lower PKC γ values than the other three groups [Fig. 3b; critical $t = 2.1, p$'s $< .05$]. Although VDAC1 was several-fold overexpressed in sedentary transgenics compared to their wild-type counterparts, none of the main or interaction effects was statistically significant [Fig. 3c; F 's $< 2.3, p$'s $> .178$]. Transgenics of both exercise groups had significantly elevated PKA2 β compared to wild-type controls [Fig. 3d; $F(1,7) = 9.9, p = .0163$]. The effect of running-wheel status and its interaction with genotype were not significant [F 's $< 0.3; p$'s $> .616$].

Table 2 shows the proteomics changes induced by long-term running activity in wild-type mice. Chronic exercise significantly altered proteins involved in oxidative phosphorylation, cytoskeletal regulation, and protein processing. Additional changes were observed in pathways known to be affected by exercise, such as glucose metabolism and synaptic transmission. Indeed, some of the same proteins altered in sedentary APP/PSEN1 transgenics in these pathways (Table 1) were affected by exercise in wild-type mice, suggesting potential avenues for improving cognition and/or attenuation of neuropathology. Interestingly, of the 30 proteins significantly overexpressed in sedentary APP/PSEN1 transgenics (Table 1), nine were reduced back to wild-type levels by voluntary exercise (Table 3). These include two uncharacterized proteins (Accession #D3Z4R5 & #F6VUT6), as well as clusters of proteins involved in neurogenesis and cytoskeletal regulation. Indeed, all four of the cytoskeletal proteins reduced in active APP/PSEN1 mice (Table 3) were significantly overexpressed in sedentary transgenics compared to sedentary wild-type mice (Table 1). None of the proteins expressed at lower levels in sedentary transgenics was significantly altered by running-wheel activity. Notably, the running-induced changes in transgenic mice were largely different from those of wild-type mice. Specifically, exercise induced broad changes in proteins associated with oxidative phosphorylation in wild-type mice, but this pathway was minimally changed in active APP/PSEN1 mice. The converse was true with respect to neurogenesis and neuroproliferation; this represented the largest cluster of proteins that were changed by exercise in transgenic mice, but there were few running-induced changes in this pathway in wild-type mice. Although proteins involved in cytoskeletal regulation were altered by running in mice of both genotypes, the effects were

in the opposite direction—largely increased for wild-type mice and uniformly decreased for transgenics. Remarkably, the disruptions in synaptic proteins observed in sedentary APP/PSEN1 transgenics were unaltered by 7 months of physical exercise. Only two proteins were affected by exercise in mice of both genotypes, glutathione s-transferase $\mu 1$ (*Gstm1*) and $\alpha 1C$ tubulin (*Tuba1c*). *Gstm1* is involved in glutathione metabolism and was downregulated 20% in active mice of both genotypes. *Tuba1c* is a cytoskeletal protein that was significantly over-expressed in sedentary transgenics (Table 1). In active mice, hippocampal *Tuba1c* levels were increased in wild-types but reduced in APP/PSEN1 transgenics. This pattern of genotypic changes was observed in a number of proteins in the cytoskeletal regulatory pathway.

Analyses of A β levels showed that active APP/PSEN1 transgenics had significantly lower total, soluble, and insoluble A β [Fig. 4a–c; λ 's < .25, F's > 7.5, p's < .0045]. Follow-up analyses showed that exercise reduced all A β measures significantly except for cortical insoluble A β 42 ($p = .389$). The average daily distance that mice ran was inversely correlated with total hippocampal A β 40 [Fig. 4d; $r(6) = -.854$, $p = .0070$] and to a lesser extent with total hippocampal A β 42 [$r(6) = -.711$, $p = .0481$]. Cortical A β levels, although significantly reduced in active transgenics, were not correlated with running (r 's < .48, p 's > .239). Hippocampal total A β 42 was positively correlated with the composite cognition score [$r(6) = .729$, $p = .0404$], but total A β 40 was unrelated [$r(6) = .448$, $p = .265$]. Neither soluble nor insoluble hippocampal A β 42 was specifically associated with memory [r 's < .631, p 's > .093]. Cortical A β levels were also unrelated to cognition in active transgenics [r 's < .501, p 's > .206], and no A β measure was significantly associated with cognition score in sedentary APP/PSEN1 mice [r 's < .709, p 's > .074]. Western blot analyses using two separate antibodies revealed that full-length APP levels were significantly increased in active APP/PSEN1 mice compared to their inactive counterparts [Fig. 4e–g; $\lambda = .07$, $F(1,2) = 26.6$, $p = .0357$]. However, BACE1 protein levels were significantly lower in active compared to sedentary transgenics ($p = .0413$), suggesting that reduced activity at the β -secretase cleavage site was at least partially responsible for reduced levels of A β (Fig. 4h). sAPP α did not differ between running-wheel groups [Fig. 4i; $F(1,4) = .0002$, $p = .989$].

Discussion

Consistent with previous reports, we show that voluntary exercise improves memory in APP-overexpressing transgenic mice and reduces A β aggregation. The cognitive deficits normally observed in APP/PSEN1 transgenics were ameliorated after 7 months of voluntary exercise. Interestingly, cognition was strongly associated with the amount of running in wild-type controls but not in transgenics. Using a classical proteomics approach, we analyzed differential protein expression associated with changes in long-term physical activity. Our data suggest that running-induced changes in proteins associated with synaptic transmission, oxidative phosphorylation, and cytoskeletal regulation in wild-type mice. Exercise altered cytoskeletal proteins in APP/PSEN1 mice as well, but in the opposite direction. Synaptic proteins were largely unaffected by exercise in the double-transgenics, but neurogenesis-related proteins were significantly altered. Soluble and insoluble A β were broadly lowered by running, and hippocampal A β was negatively correlated with exercise levels. Lower levels of β -secretase in active APP/PSEN1 mice may have contributed to the

reduced A β . Together, these results suggest that exercise may improve cognition in fundamentally different ways in wild-type and APP/PSEN1 transgenic mice.

Studies examining the effects of exercise on A β -associated neuropathology have produced a variety of results. This is not surprising given the different mutant lines, promoters, background strains, exercise regimens, age at initiation, diet, experimental environments, and housing conditions. Nevertheless, some exercise-induced changes occur with sufficient frequency in spite of methodological differences that they may inform us about the most robust and reliable effects. Chief among them is a reduction in A β and plaque formation. Consistent with most studies of voluntary exercise, we demonstrate significantly decreased A β in active transgenic mice. Although full-length APP protein levels were elevated in the active transgenics relative to sedentary controls, BACE1 protein was significantly reduced. This suggests that reduced amyloidogenic β -secretase cleavage in active APP/PSEN1 transgenics was at least in part responsible for the lower A β levels. To our knowledge this is the first report of exercise-induced changes in APP or BACE1 in APP-overexpressing mice. Three studies reported decreased α - and β -CTFs following voluntary exercise in TgCRND8 or PDGF transgenics but normal α -, β -, and γ -secretase activity (Adlard et al., 2005; Maesako et al., 2013; Maesako et al., 2012). Maesako et al. also reported increased activity of neprilysin, suggesting increased degradation of A β . Thus there may be multiple mechanisms by which exercise can reduce A β .

Many of the genotype differences noted in sedentary mice are established characteristics of APP-overexpressing mice are associated with impaired mitochondrial dysfunction and calcium dysregulation. Changes in five proteins suggest abnormal oxidative phosphorylation, which can result from mitochondrial dysfunction. This in turn alters the tricarboxylic acid cycle and directly targets A β (Blass, 2000; Karuppagounder et al., 2009). Synaptic proteins were also significantly altered. VDAC1 is a γ -secretase-associated protein localized to detergent-resistant membranes (DRMs), concentrated in synapses and the outer mitochondrial membrane (Hur et al., 2012; Sampson et al., 1996; Yoo et al., 2001). DRMs are rich in gangliosides and cholesterol, and the site of APP processing and A β aggregation (Ariga et al., 2008, 2010, 2013). Consistent with this, VDAC1 is associated with increased production of A β 40 and A β 42 and is elevated in brains of Alzheimer patients and APP-overexpressing transgenic mice (Cuadrado-Tejedor et al., 2011; Ramirez et al., 2009; Reddy, 2013; Yoo et al., 2001). In one study, shRNA-mediated knock-down of VDAC1 reduced A β levels by 70% (Hur et al., 2012). Thus it is not surprising that VDAC1 was increased in the sedentary transgenics relative to wild-type mice. The fact that physical exercise did not significantly alter VDAC1 or other synaptic proteins suggests that this did not substantively contribute to the reduced A β or improved cognition observed in the active APP/PSEN1 mice.

When allowed to run freely, wild-type mice exhibited protein changes associated with enhanced cellular function, energy metabolism, and ATP production. Notably, all seven of the proteins appearing in both Tables 1 and 2 changed in the same direction, i.e., those elevated in sedentary transgenics were increased by running in wild-type mice, and those reduced in sedentary transgenics were decreased by running in wild-type mice. This suggests putative compensatory processes in the sedentary transgenics that were not

sufficient to ameliorate the cognitive deficits. With respect to cognition, the most relevant protein changes in wild-type mice were those involved with synaptic transmission. Cognition was tightly associated with running in wild-type mice but uncorrelated in transgenics. In contrast to robust changes observed in wild-type mice, synaptic proteins were scarcely changed by exercise in transgenic mice. This is consistent with a number of papers showing an inability of APP-overexpressing mice to enhance pathways associated with synaptic plasticity in response to increased physical or cognitive demands (Caccamo et al., 2010; Dineley et al., 2010; Hu et al., 2013).

In contrast to synaptic proteins, running reversed many of the other protein abnormalities in the transgenic mice. Table 1 shows that cytoskeletal proteins may be unstable in sedentary APP/PSEN1 mice. When the transgenics were allowed to exercise, we observed a shift toward stable expression of these proteins. Cytoskeletal integrity is important for proper memory function, and is disrupted in Alzheimer's disease (Castegna et al., 2002; Castegna et al., 2003). Thus the normalization of these proteins by exercise in the present study may have contributed to the cognitive improvement in the APP/PSEN1 transgenics. Proteins associated with neuroproliferation and neurogenesis also returned to wild-type levels in APP/PSEN1 transgenics allowed to exercise. In a number of mouse lines harboring human APP and PSEN1 transgenes, neuroproliferation, neurogenesis, and survival of new neurons are diminished relative to wild-type controls (Biscaro et al., 2009; Biscaro et al., 2012; Hamilton and Holscher, 2012; Li et al., 2014). In contrast, proliferation of neural stem cells (NSCs) is reported to be either normal or elevated in the APP/PSEN1 transgenic line used in the present study (He et al., 2013; Kamphuis et al., 2012; Niidome et al., 2008; Taniuchi et al., 2007). However, the NSCs in this transgenic line are fated to become glia and the number of new neurons generated is reduced relative to wild-type controls (Lazarov and Marr, 2010; Winner et al., 2011).

With exercise, these proteins are returned to wild-type levels, suggesting that neuroproliferation has normalized. Impaired neurogenesis can also be induced by intracranial injections of A β 42 (Zheng et al., 2013). Thus the normalization of proteins associated with neuroproliferation and neurogenesis in the active PSEN1 transgenics may be secondary to the reduction in A β 42.

Total hippocampal A β 42 was positively associated with cognition in active transgenics despite markedly lower levels overall in this group and a negative correlation with the amount of exercise. This is counter-intuitive given the well-established role of small A β 42 oligomers in disrupting synaptic architecture and plasticity (Klyubin et al., 2005; Marcello et al., 2007; Selkoe, 2008). However, there is some indication that endogenous A β may serve a neuroprotective role, given that it is generated in response to toxic conditions such as oxidative stress or hypoxia (D'Alton and George, 2011). Indeed, a number of studies have shown that A β monomers are neuroprotective and improve memory (Giuffrida et al., 2009; O'Nuallain et al., 2011; Scopes et al., 2012). A β 42 may enhance cognition through its ability to facilitate neurogenesis at low concentrations. Sotthibundhu et al. (Sotthibundhu) demonstrated concentration-dependent enhancement of neurogenesis by oligomeric A β 42 *in vitro* up to a marginally-toxic 5 μ M. *In vivo*, they showed that intraventricular injections of low concentrations of A β 42, but not A β 16, increased neurogenic activity in the

subventricular zone of wild-type mice. Others have shown that this enhancement is specific to the 1–42 sequence and is not observed with 1–40 or 25–35 (Calafiore et al., 2006; Heo et al., 2007; Jin et al., 2004; López-Toledano and Shelanski, 2004). Our data are consistent with this. In sedentary transgenics whose A β 42 levels were high, memory was poor. When A β 42 concentrations were lower following exercise, proteins associated with neurogenesis were normalized and A β 42 was positively correlated with cognition. In addition to the positive effects of A β 42, APP may have exerted beneficial effects on the cognitive status of the active transgenics. Elevated 3–4 fold in active transgenics, APP has been shown to protect against synaptic damage, excitotoxicity, and oxidative stress in the absence of high levels of A β (Masliah et al., 1997; Mucke et al., 1994).

Our data illustrate several potential avenues for mechanistic investigation into the ways in which voluntary physical exercise may ameliorate Alzheimer-like neuropathology and improve memory. The enhancement of synaptic plasticity by exercise has been demonstrated previously, and may have played a role in the cognitive performance of wild-type mice in the present study. In contrast, synaptic proteins did not appreciably change in the active transgenics. Instead, the large improvement in memory in these mice was associated with reduced A β and normalization of proteins involved in neurogenesis and cytoskeletal regulation. Unlike previous reports, the exercise-induced A β reductions in the present study were accompanied by lower β -secretase levels. Taken together, these data show that multiple mechanisms may exist by which running may reduce A β , but that some well-established abnormalities exhibited by these double-transgenics, such as synaptic transmission and calcium dysregulation, are not substantially altered by 7 months of exercise.

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Highlights

- Voluntary exercise improved cognition in APP/PSEN1 double-transgenic mice.
- The distance run was correlated with memory in wild-type ($r^2 = .96$) but not APP/PSEN1 ($r^2 = .01$) mice.
- Proteomics revealed genotype differences in sedentary mice and exercise-induced changes.
- Soluble and insoluble A β 40 and A β 42 levels were significantly reduced in active transgenics.
- Exercise increased full-length APP and decreased BACE1 in APP/PSEN1 mice.

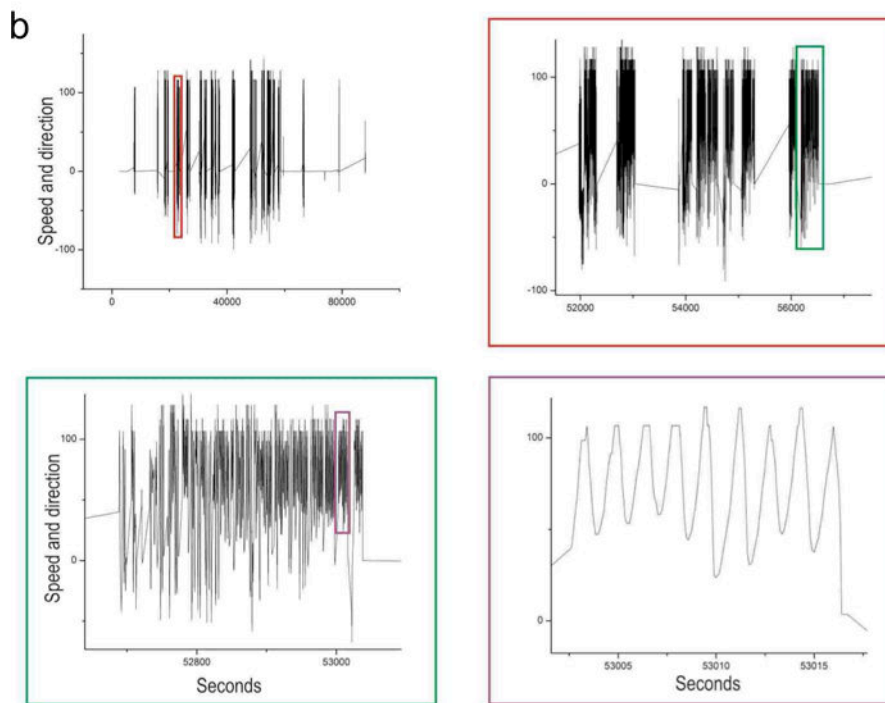


Figure 1. Novel running-wheel system
 (a) Commercially-available running wheels were adapted for novel home-made electronics that allow quick cage change, and software that eliminates movement due to rocking and adventitious sensor proximity. (b) Four panels document with increasing precision the readout from the running-wheel software.

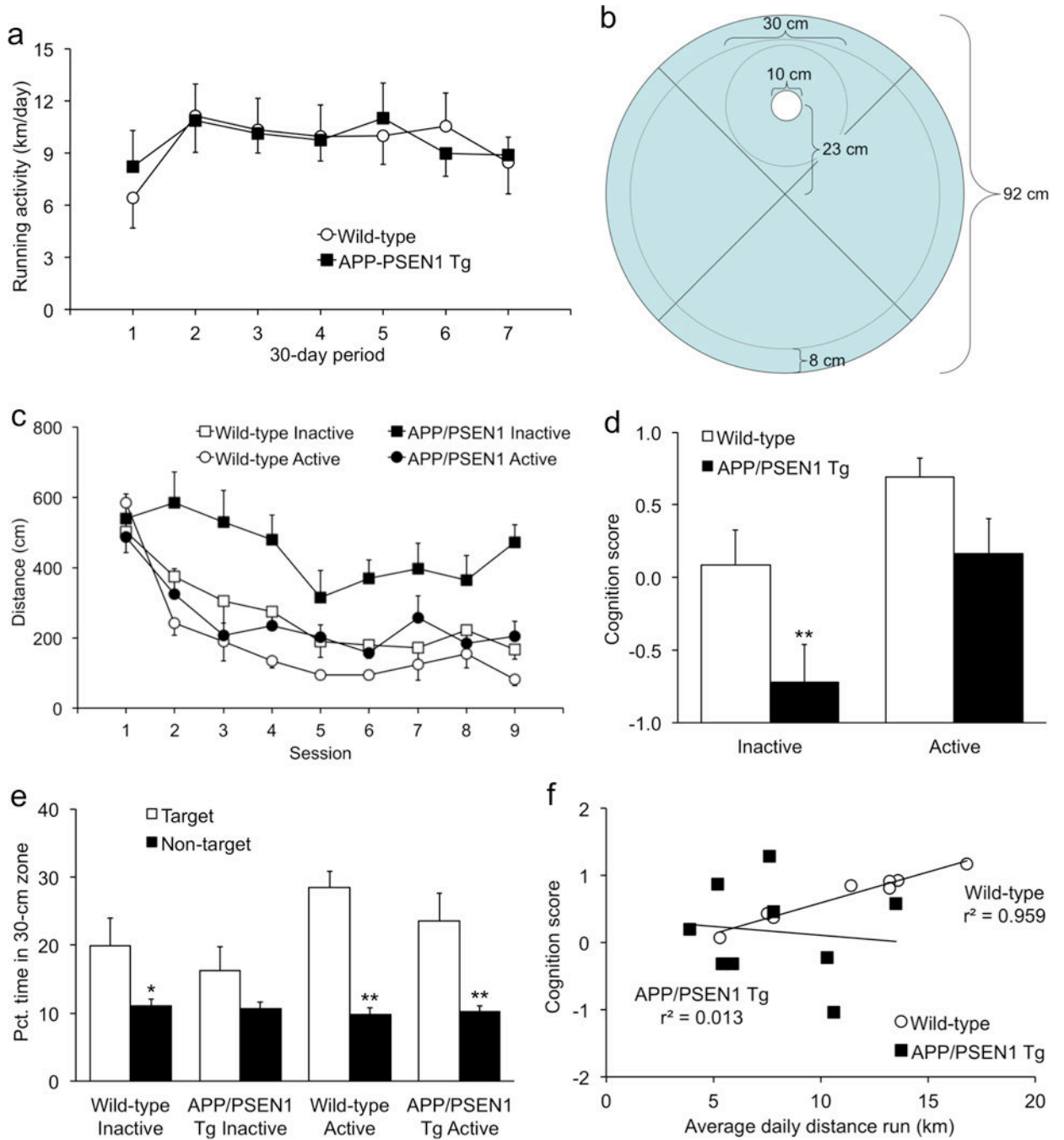


Figure 2. Cognitive performance is tightly associated with running in wild-type mice but not APP/PSEN1 transgenics

(a) During the first month of access to the running wheel, APP/PSEN1 transgenic mice ran nearly twice as much as wild-type control mice, although the difference was not statistically significant. For the rest of the study, wild-type mice increased their output to transgenic levels. (b) Diagram of water maze configured for more precise measurement of memory. A 30-cm zone is used to measure memory for the platform location. (c) Sedentary APP/PSEN1 transgenics had significantly impaired spatial learning compared with wild-type controls.

Transgenics allowed to exercise freely had significantly improved performance. **(d)** A cognition score calculated from asymptotic performance during acquisition and precision during the probe trial was significantly lower in sedentary transgenic mice. **(e)** Sedentary transgenic mice did not spend more time in the target 30-cm zone than in the mean of equivalent zones in the other three quadrants. In contrast, mice in the other three groups all showed selective search for the target zone. **(f)** The cognition scores were significantly correlated with running in wild-type mice, but not in APP/PSEN1 transgenics. * $p < .05$; ** $p < .01$.

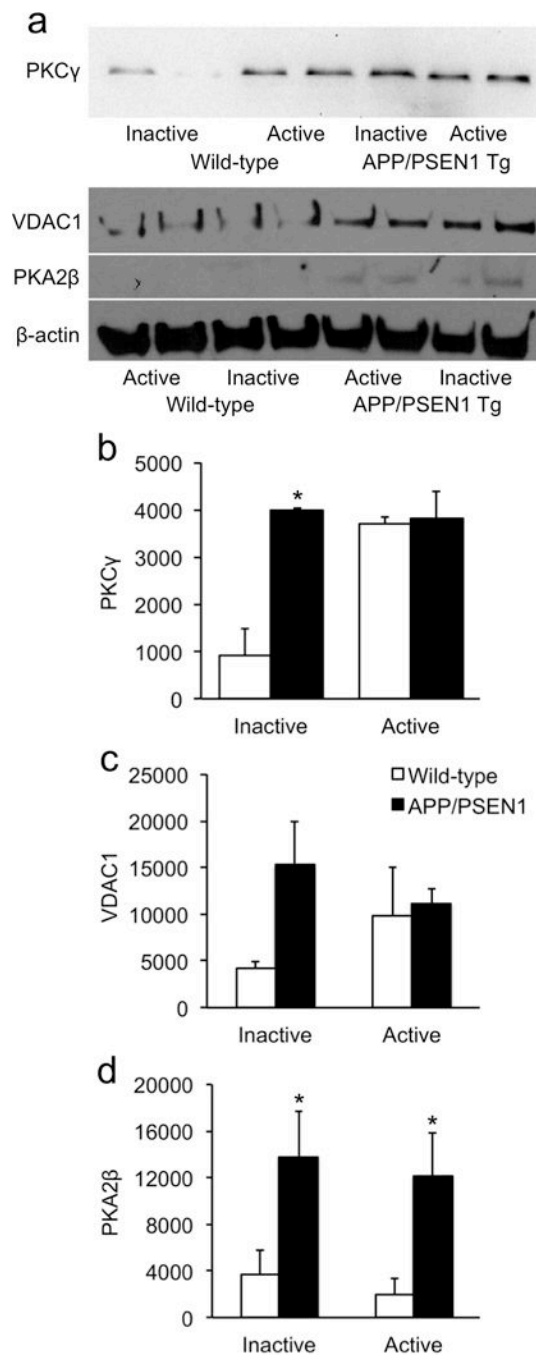


Figure 3. Validation of highly-overexpressed proteins in sedentary APP/PSEN1 transgenic mice (a) Western blots of three of the most over-expressed proteins from the proteomics data in Table 1. Protein levels were elevated in sedentary transgenics compared to sedentary wild-type controls. (b) PKC γ was significantly elevated in sedentary transgenic mice. In response to exercise, PKC γ levels increased in wild-type mice but were unchanged in transgenics. (c) VDAC1 was elevated several fold in sedentary APP/PSEN1 mice compared to sedentary wild-types, although the difference was not statistically significant. (d) PKA2 β was

significantly elevated in both transgenic groups, compared to their wild-type counterparts.
*p < .05.

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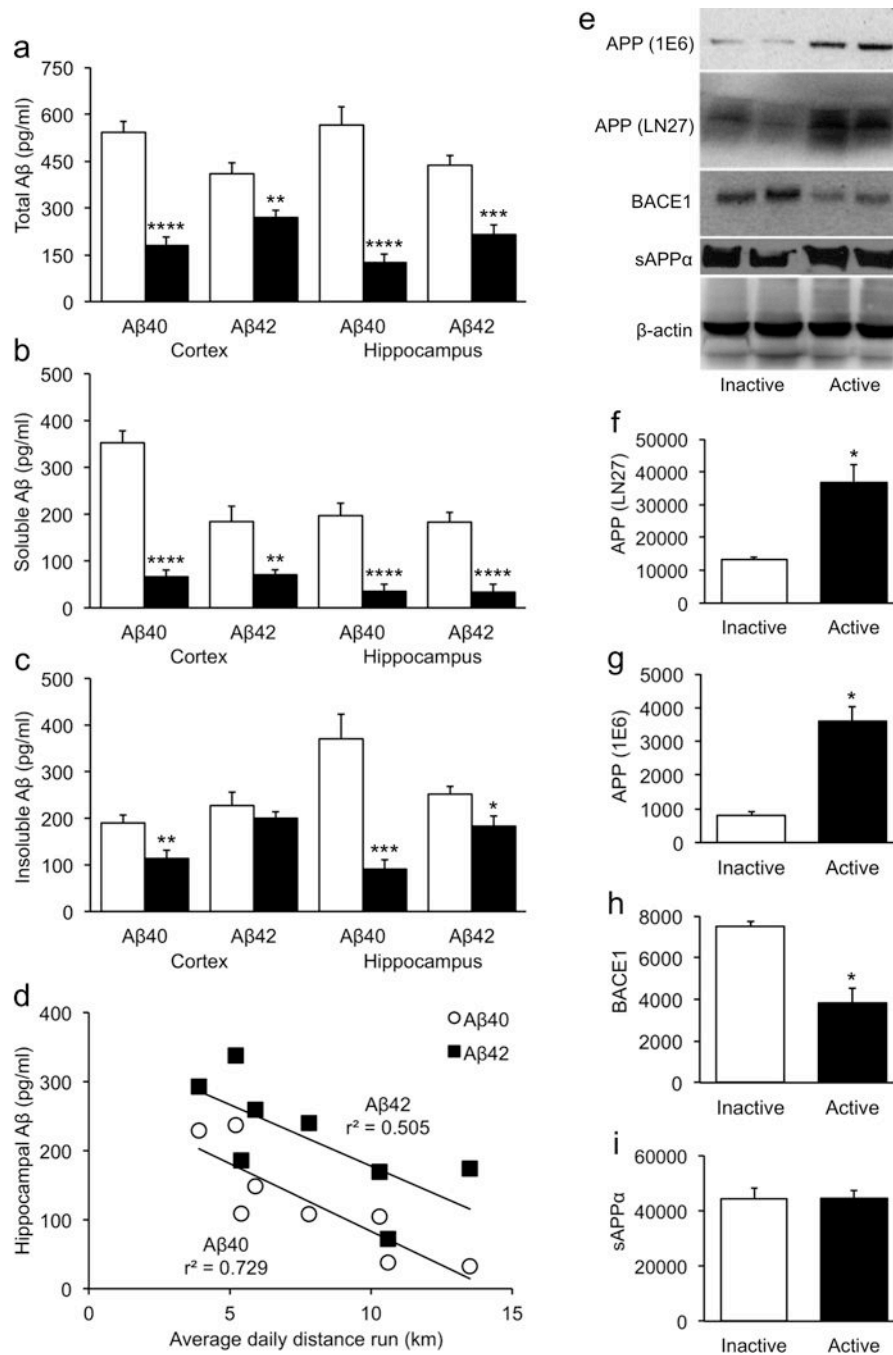


Figure 4. Exercise-induced reductions in Aβ are associated with reduced BACE1
(a–c) Voluntary exercise reduced both soluble and insoluble Aβ in APP/PSEN1 transgenics, in both in hippocampus and overlying cortex. **(d)** The reduction in Aβ in active transgenics was significantly correlated with the amount of running. Running was not associated with cortical Aβ levels. **(e–g)** Full-length amyloid precursor protein (APP) was measured using two different antibodies. Both immunoblots indicated that APP was significantly elevated in active APP/PSEN1 mice. **(e,h–i)** In contrast to APP, BACE1 was significantly reduced in

active transgenics. (i) Levels of sAPP β were unchanged in active transgenics, compared to their sedentary counterparts. * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$.

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Table 1
 Proteins differentially expressed between Inactive APP/PSEN1 and Inactive Wild-type (control) mice.

Accession number	Protein	Gene	Mol. wt. (kDa)	Peptides	Fold change	p	Pathway
Q2NKL4	Protein kinase C γ (PKC γ)	Pkcg	72.9	8	10.0	<.05	Synaptic transmission
Q9CZV8	F-box/LRR-repeat 20	Fbxl20	48.3	2	3.6	<.05	
Q3TYH2	Ras-related protein Rab-15	Rab15	24.3	3	2.9	<.05	
A2ALV3	Endophilin-A1	Sh3gl2	48.2	10	1.7	<.01	
Q9JIS5	Synaptic vesicle glycoprotein 2A	Sv2a	82.6	8	1.7	<.05	
B0QZN5	Vesicle-associated membrane 2	Vamp2	17.8	3	1.6	<.05	
P80316	T-complex protein 1 subunit ϵ	Cct5	59.6	3	2.9	<.05	Cytoskeletal regulation
A2ASS6	Titin	Ttn	3904.0	34	2.1	<.01	
Q3TGW0	Actin-related protein 3	Actr3	47.3	8	1.6	<.05	
Q3TIZ0	Tubulin α 1C	Tubal1c	49.9	22	1.2	<.05	
Q6ZWR6	Nesprin-1	Syne1	1009.3	7	-	<.01	
E9Q7P1	Collagen, type XXII, α 1	Col22a1	159.8	2	-	<.01	
B3KXD8	Formin-like 3	Fnnl3	66.7	2	0.8	<.05	
Q9JKC6	Cell cycle exit and neuronal differentiation 1	Cend1	15.0	2	8.6	<.01	Neurogenesis/neuroproliferation
Q61488	Desert hedgehog	Dhh	43.5	2	5.7	<.05	
P70388	DNA repair RAD50	Rad50	153.4	3	-	<.05	
E9QKH0	CLIP-associating protein 1 isoform 1	Clasp1	169.2	4	0.5	<.05	
P04259	Keratin, type II cytoskeletal 6B (cytokeratin 6B)	Krt6b	60.0	11	0.3	<.05	
B4E1T1	Keratin, type II cytoskeletal 5 (cytokeratin 5)	Krt5	58.8	10	0.3	<.05	
Q3TL54	Tripartite motif-containing 43A	Trim43a	52.1	3	5.7	<.05	Protein processing
Q9ER38	Torsin-3A	Tors3a	43.8	2	-	<.01	
Q3TIF8	40S ribosomal S24	Rps24	15.4	2	-	<.05	
Q9D2H5	Tripartite motif-containing 42	Trim42	82.9	2	0.3	<.05	
Q6ZPR1	Cilia and flagella associated protein 97	Kiaa1430	60.8	2	0.0	<.05	
P31324	cAMP-dependent protein kinase II- β regulatory subunit (PKA2 β)	Pkar2b	46.1	4	18.6	<.001	Apoptosis
P97350	Plakophilin-1	Pkpl	80.8	5	5.2	<.01	
P68254	14-3-3 θ	Ywhaq	27.7	12	0.6	<.01	
Q3THL7	Voltage-dependent anion channel 1	VDAC1	30.7	6	20.0	<.001	Oxidative phosphorylation

Accession number	Protein	Gene	Mol. wt. (kDa)	Peptides	Fold change	P	Pathway
Q9D6R2	Isocitrate dehydrogenase [NAD] subunit α , mitochondrial	Ihd3a	39.6	9	2.8	<01	
P14094	Sodium/potassium-transporting ATPase subunit β -1	Atp1b1	35.2	10	1.3	<.05	
Q3THG0	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	Atp5o	23.4	4	-	<.01	
Q68FL4	Adenosylhomocysteinase 3	Ahcy12	66.8	4	0.2	<.05	Glycolysis/gluconeogenesis
O70250	Phosphoglycerate mutase 2	Pgam2	28.8	2	3.4	<.01	
P40142	Transketolase	Tkt	67.6	14	0.7	<.05	
P45376	Aldose reductase	Akr1b1	35.7	3	0.2	<.01	
P03995	Glial fibrillary acidic protein	Gfap	49.9	20	12.1	<.01	Neuroinflammation
P07901	Heat shock 90- α	Hsp90aa1	84.7	35	0.7	<.01	
D3Z4R5	Uncharacterized		42.4	4	5.7	<.05	Unknown
F6WSS0	GTP-binding protein 10	Gtpbp10	8.3	2	3.6	<.05	
D3YX13	uncharacterized 3		50.0	16	1.2	<.05	
F6VUT6	Protein Gm8251	Gm8251	225.8	3	-	<.05	
D3Z4Y4	Keratin 5	Krt5	60.3	9	0.4	<.01	

All proteins are listed that had significantly different main or interaction effects using 2×2 ANOVAs followed by Fisher's protected LSD; df = 15 for each comparison. A 0-fold change indicates that the protein was not expressed at detectable levels in the inactive APP/PSEN1 mice; a dash indicates that the protein was not expressed at detectable levels in the inactive wild-type mice.

Table 2

Proteins differentially expressed between Active and Inactive (control) Wild-type mice.

Accession number	Protein	Gene	Mol. wt. (kDa)	Peptides	Fold change	p	Pathway
Q3TTA7	E3 ubiquitin ligase CBL-B	Cblb	109.2	3	7.1	<.001	Oxidative phosphorylation
Q9D6R2	Isocitrate dehydrogenase [NAD] subunit α , mitochondrial	Idh3a	39.6	9	2.8	<.01	
Q60597	2-oxoglutarate dehydrogenase, mitochondrial	Ogdh	116.4	7	1.9	<.05	
O08749	Dihydropyrimidine dehydrogenase, mitochondrial	Dld	54.2	12	1.7	<.05	
Q99K10	Aconitate hydratase, mitochondrial	Aco2	85.4	34	0.8	<.05	
P97807	Fumarate hydratase, mitochondrial	Fh	54.3	13	0.7	<.01	
A2AKV3	ATP synthase, γ subunit, mitochondrial	Atp5c1	20.6	6	0.3	<.05	
Q8BUR4	Dedicator of cytokinesis 1	Dock1	214.9	3	5.7	<.05	Cytoskeletal regulation
Q8C928	Erythrocyte protein band 4.1-like 2	Epb4.1l2	89.8	4	5.0	<.05	
Q3U9U3	Tubulin, β class V	Tubb6	42.1	13	1.4	<.05	
Q3TIZ0	Tubulin, α 1C	Tuba1c	49.9	22	1.1	<.05	
Q8CEH8	Profilin 1	Pfn1	14.9	6	0.5	<.05	
Q8BWQ5	Serine/threonine kinase DCLK3	Dclk3	88.2	3	8.6	<.01	Protein processing
Q9D2F7	Nuclear pore membrane glycoprotein 210-like	Nup210l	208.6	2	-	<.01	
A2AH75	Kinesin family member 1B	Kif1b	130.0	3	-	<.05	
Q9D2H5	Tripartite motif-containing protein 42	Trim42	82.9	2	0.3	<.05	
Q2NKI4	Protein kinase C γ (PKC γ)	Prkcg	72.9	8	12.9	<.05	Synaptic transmission
P04627	Serine/threonine kinase A-Raf	Araf	67.5	2	-	<.05	
P01831	Thy-1 membrane glycoprotein	Thy1	18.1	5	0.3	<.01	
P45376	Aldose reductase	Akr1b1	35.7	3	0.5	<.05	Glycolysis/gluconeogenesis
O89116	Vesicle transport through interaction with t-SNAREs homolog 1A	Vti1a	25.0	2	8.6	<.01	Ion Transport
A8DUV1	α -globin	Hbat1	15.1	6	1.5	<.05	
Q8VH31	Ret finger protein-like 4A	Rfpl4a	32.3	2	-	<.05	
Q91KC6	Cell cycle exit and neuronal differentiation 1	Cend1	15.0	2	22.9	<.001	Neurogenesis neuroproliferation
E9QKH0	CLIP-associating protein 1	Clasp1	169.2	4	0.4	<.05	
P10649	Glutathione S-transferase μ 1	Gstm1	25.9	16	0.8	<.05	Glutathione metabolism
O70472	Transmembrane protein 131	Tmem131	200.3	2	-	<.05	Neuroinflammation
B7U582	Heat shock protein 70-2	Hspa2	69.7	11	0.8	<.05	MAPK/P13K signaling

Accession number	Protein	Gene	Mol. wt. (kDa)	Peptides	Fold change	p	Pathway
Q9CQQ8	U6 snRNA-associated Sm-like LSm7	Lsm7	11.6	2	9.3	<.01	RNA degradation
Q8BGHI	Uncharacterized		73.3	2	3.6	<.05	Unknown

All proteins are listed that had significantly different main or interaction effects using 2×2 ANOVAs followed by Fisher's protected LSD; df = 13 for each comparison. A 0-fold change indicates that the protein was not expressed at detectable levels in the active wild-type mice; a dash indicates that the protein was not expressed at detectable levels in the inactive wild-type mice.

Table 3

Proteins differentially expressed between Active and Inactive (control) APP/PSEN1 mice.

Accession number	Protein	Gene	Mol. wt. (kDa)	Peptides	Fold change	p	Pathway
A3KFM7	Chromodomain helicase DNA binding 6	Chd6	305.2	7	-	<.01	Neurogenesis/neuroproliferation
P97477	Aurora kinase A	Aurka	47.1	4	-	<.05	
Q3UJT6	Replication factor C (activator 1)	Rfc1	64.6	3	-	<.05	
Q64522	Histone H2A type 2-B	Hist2h2ab	14.0	3	0.5	<.05	
Q61488	Desert hedgehog	Dhh	43.5	2	0.0	<.01	
P70388	DNA repair RAD50	Rad50	153.4	3	0.0	<.05	
B1AY10	Transcriptional repressor NF-X1	Nfx1	123.7	4	0.0	<.05	
Q3TIZ0	Tubulin α 1C	Tubalc	249.9	2	0.9	<.05	Cytoskeletal regulation
Q3TGW0	Actin-related 3	Actr3	47.3	8	0.6	<.05	
P80316	T-complex protein 1 subunit ϵ	Cct5	59.6	3	0.2	<.05	
E9Q7P1	Collagen, type XXII, α 1	Col22a1	159.8	2	0.1	<.05	
Q55XR6	Clathrin, heavy chain	Cltc	9191.8	91	0.8	<.05	Endocytosis
Q80Z16	E3 ubiquitin ligase LRSAMI	Lrsam1	83.9	2	0.0	<.05	
Q8CBD1	Nuclear receptor-interacting protein 1	Nrip1	126.2	2	-	<.05	Protein processing
A2RT70	Tenascin-R	Tnr	1149.5	11	4.2	<.01	Neuritogenesis
Q3T143	Vacuolar protein sorting-associated protein 35	Vps35	91.6	7	3.2	<.05	
P08249	Malate dehydrogenase, mitochondrial	Mdh2	135.6	6	1.3	<.01	Oxidative phosphorylation
P14094	Sodium/potassium-transporting ATPase subunit β -1	Atp1b1	135.2	10	0.7	<.001	
P10649	Glutathione S-transferase μ 1	Gstm1	125.9	6	0.8	<.05	Glutathione metabolism
E9Q3B9	Monoglyceride lipase	Mgll	35.2	2	0.0	<.05	Glycerolipid metabolism
B4DZ37	Solute carrier family 9, isoform 10	Slc9a10	51.3	2	0.0	<.05	Ion transport
A2ANU2	Polypeptide N-acetylgalactosaminyltransferase 12	Galnt12	33.1	2	3.9	<.05	O-Glycan biosynthesis
D0FYX1	Peptidase S1 and S6, chymotrypsin/Hap		51.5	2	3.5	<.05	Unknown
Q6PNC0	Dmx-like protein 1	Dmx11	335.8	4	-	<.05	
D3Z7K0	Ubiquitin thioesterase	Outb1	11.2	5	0.3	<.05	
D3Z4R5	Uncharacterized		42.4	4	0.0	<.01	
F6VUT6	Protein Gm8251	Gm8251	225.8	3	0.0	<.05	

All proteins are listed that had significantly different main or interaction effects using 2×2 ANOVAs followed by Fisher's protected LSD; $df = 13$ for each comparison. A 0-fold change indicates that the protein was not expressed at detectable levels in the active APP/PSEN1 mice; a dash indicates that the protein was not expressed at detectable levels in the inactive APP/PSEN1 mice.

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