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Physical Activity-Associated Gene Expression Signature in Nonhuman Primate Motor Cortex

Amanda C. Mitchell^{1,2}, Rehana K. Leak^{3,4}, Krassimira Garbett^{1,2}, Michael J. Zigmond⁴, Judy L. Cameron^{5,6}, and Károly Mirnics^{1,2}

¹Department of Psychiatry Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, Tennessee, USA

²Department of Vanderbilt Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, Tennessee, USA

³Division of Pharmaceutical Sciences, Mylan School of Pharmacy, Duquesne University, Pittsburgh, Pennsylvania, USA

⁴Department of Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

⁵Department of Psychiatry, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

⁶Oregon National Primate Research Center, Beaverton, Oregon, USA

Abstract

It has been established that weight gain and weight loss are heavily influenced by activity level. In this study, we hypothesized that the motor cortex exhibits a distinct physical activity-associated gene expression profile, which may underlie changes in weight associated with movement. Using DNA microarrays we profiled gene expression in the motor cortex of a group of 14 female rhesus monkeys (*Macaca mulatta*) with a wide range of stable physical activity levels. We found that neuronal growth factor signaling and nutrient sensing transcripts in the brain were highly correlated with physical activity. A follow-up of AKT3 expression changes (a gene at the apex of neuronal survival and nutrient sensing) revealed increased protein levels of total AKT, phosphorylated AKT, and forkhead box O3 (FOXO3), one of AKT's main downstream effectors. In addition, we successfully validated three other genes via quantitative polymerase chain reaction (qPCR) (cereblon (CRBN), origin recognition complex subunit 4-like, and pyruvate dehydrogenase 4 (PDK4)). We conclude that these genes are important in the physical activity-associated pathway in the motor cortex, and may be critical for physical activity-associated changes in body weight and neuroprotection.

Physical activity plays a critical role in energy balance and weight loss. Looking in our cohort of adult rhesus monkeys (*Macaca mulatta*) with a wide range of physical activity levels, we previously established that physical activity influences both weight gain and weight loss. The most active monkeys gained the least amount of weight as a part of a weight gain study (1), and as part of a weight loss study, the animals who did not lose weight compensated for their reduced food intake with a decrease in their activity level (2).

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Correspondence: Karoly Mirnics, karoly.mirnics@vanderbilt.edu.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/oby>

DISCLOSURE

The authors declared no conflict of interest.

These studies suggest that physical activity levels must be increased to lose significant weight in adulthood.

In addition to its role in the regulation of body weight, physical activity also is associated with a decreased lifetime risk of neurological disorders, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (3–6). In the brain, physical activity most dramatically affects the hippocampus (7) with increases in neurogenesis (8), nerve growth factor, and brain derived neurotrophic factor (7). In contrast, little attention has been paid to other brain regions that undoubtedly play a role in the interaction between physical activity and neurological disease. The motor cortex is one such area. Motor neurons in this region are devastated in amyotrophic lateral sclerosis (6). Moreover, the motor cortex is affected with synuclein and tau pathology at the end stages of Parkinson's and Alzheimer's disease (9), and physical activity is known to slow the progress of both disorders.

Based on these studies, we hypothesized that spontaneous physical activity is associated with a unique gene expression signature in the motor cortex that may be important in body weight regulation as well as protection against neurodegenerative disease. To test this hypothesis, we performed whole genome expression profiling of the motor cortex of 14 female rhesus monkeys with a wide range of stable physical activity levels using both Affymetrix Rhesus Macaque Genome (RhG) and human genome (HG) microarrays (Figure 1). Physical activity levels ranged from 46,376 counts per day to 897,948 counts per day with a skew of 1.93, indicating that our activity measurements were skewed right with more animals having low activity values. The rhesus and human genomes share 93% sequence identity (10) and we have successfully used human microarrays to query rhesus monkey gene expression changes in the past (11). Whereas the Affymetrix RhG microarrays provided us with the most specific hybridization information for rhesus monkeys, the Affymetrix HG microarrays contained more extensive and higher quality annotations.

Microarray data were $\log_2$ transformed, robust multi-array analysis-normalized (12,13), and linearly modeled with physical activity level. We focused on convergent findings between the RhG and HG microarrays, which enabled us to identify a strong and distinct physical activity-associated gene expression signature of 98 significantly altered transcripts (Supplementary Table S1 online). Ninety-six of the 98 significantly altered transcripts changed in the same direction on both microarrays, suggesting a high degree of concordance between the two data sets and a low false discovery rate. Furthermore, our Pearson Hierarchical Clustering (14) revealed a progression of gene expression levels from monkeys with low activity to monkeys with high activity (Figure 2).

Gene set enrichment analysis returned no significantly altered pathways in our dataset, but several genes with altered transcript levels were part of signaling cascades and nutrient sensing pathways. Namely, AKT3, which showed more than a twofold change in expression over the quartile in our dataset, is at the apex of both growth factor signaling and nutrient sensing (15,16). AKT pathway activation is associated with physical activity in the hippocampus (17,18). When activated, it phosphorylates downstream effectors, such as glycogen synthase kinase (which when inhibited phosphorylation promotes glycogen synthesis (19)), tuberous sclerosis protein 2 (which activates mammalian target of rapamycin complex 1 and is involved in ribosome biogenesis and protein synthesis (15)), and forkhead box O3 (FOXO3) (which when phosphorylated is no longer recruited to the nucleus where it would bind as a transcription factor to apoptotic related genes (17)).

To validate the correlation of AKT3 gene expression level with physical activity we performed quantitative polymerase chain reaction (qPCR) on 12 of the monkeys studied by microarrays. In addition, we also assessed the expression-activity correlation for two other

nutrient genes, cereblon (CRBN) and pyruvate dehydrogenase 4 (PDK4), and origin recognition complex subunit 4-like (Figure 3). CRBN and PDK4 converge on AMP activated protein kinase (AMPK), the master energy sensor. During exercise AMPK activity is known to increase and promote catabolic processes and inhibit anabolic processes thereby increasing cellular energy. CRBN decreases phosphorylation of the $\alpha 1$ subunit of AMPK to inhibit its activity (20), while PDK4 is induced by AMPK activity (21). The qPCR validation revealed that AKT3, CRBN, and PDK4 and origin recognition complex subunit 4-like showed positive correlation with increasing physical activity.

Furthermore, we examined AKT protein expression, its phosphorylation state at serine 473, which is required for its full activation (15), and the protein expression of its down-stream effector, FOXO3. While total AKT (tAKT) levels were not changed, we found a significant and positive correlation of active pAKT ($R = 0.624$, $P = 0.017$ for pAKT/tAKT, and $R = 0.542$, $P = 0.045$ for pAKT/actin) with physical activity level (Figure 4a). We also found a significant and positive correlation of FOXO3 protein level with spontaneous activity levels ($R = 0.649$, $P = 0.012$) (Figure 4b). Given the striking changes in mRNA levels of AKT3, it seems likely that the immunoblotting data were also assessing changes in this protein. However, we used pan-AKT antibodies that could not distinguish between the different AKT isoforms *per se*. Nonetheless, our data suggest AKT phosphorylation might be a critical molecular effector of physical activity in the motor cortex.

In summary, our study reveals that (i) spontaneous physical activity in Rhesus monkeys is associated with a strong and reproducible gene expression signature in the motor cortex, (ii) the observed gene expression signature is related to activation of transcriptional machinery, and (iii) spontaneous activity is associated with activation of the AKT-FOXO3 signaling pathway and several nutrient sensing transcripts. Given previous findings in the hippocampus, it is noteworthy that we did not observe changes in growth factor or growth factor receptors in the motor cortex, suggesting that spontaneous activity levels and planned exercise might have distinct gene expression profiles across the different brain regions. Whereas planned exercise might be growth factor dependent (and activate the AKT pathway), it is conceivable that growth factor systems still could be activated by nontranscriptional cellular machinery or that spontaneous activity induces AKT/FOXO3 through a different, nongrowth factor dependent pathway, but this deserves further investigation. However, our studies did not examine the causality of these changes, but the strong correlational relationship suggests that this is an important avenue for further investigations.

Studies in knockout mice suggest that the three isoforms of AKT play vastly differing roles: mice without AKT1 have decreased body weight, whereas mice without AKT2 have increased fasting plasma glucose levels and diabetes symptoms while mice without AKT3 have decreased brain weight (22,23). These data also suggest that AKT3 might be critical for neuronal health, and raise the possibility that activation of the AKT3 signaling pathway may play a crucial role in activity-induced weight loss and the delayed onset of neurological disorders associated with physical activity. Indeed, we have previously found that both physical activity and the neurotrophic factor GDNF increase the phosphorylation state of AKT in the rodent striatum (AD. Smith, SL. Castro, N. Lindgren, and M.J.Z., unpublished data). Thus, future studies are warranted to address whether varying levels of spontaneous motor activity are associated with differences in vulnerability to disease models and whether protection against such models can be attenuated by inhibition of AKT signaling.

RESEARCH METHODS AND PROCEDURES

Subjects

The Animal Care and Use Committee of the Oregon National Primate Research Center reviewed and approved all experiments. Fourteen adult female ovariectomized Rhesus monkeys (*M. mulatta*) with various levels of spontaneous activity were used in this study with care methods previously published (1,2,24). Their activity levels were measured in counts/ day with an accelerometer throughout the study and ranged from 46,378 to 897,948 activity counts per day. These monkeys also participated in a previous weight loss study (1). Monkeys were deeply anesthetized with ketamine/pentobarbital, quickly decapitated, and the entire brain was removed from the skull. The brain was then hemisected by a midline sagittal cut. The right hemisphere was cut into 5-mm thick coronal blocks, flash-frozen in isopentane over dry ice, and stored at -80°C until use (25).

Sample preparation, hybridization, and DNA microarray processing

Motor cortex was dissected from frozen coronal blocks using anatomical landmarks, homogenized and total RNA isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) with RNA quality assessed via analysis on an Agilent 2100 Bioanalyzer. Only samples with an RNA integrity number >7.0 were considered for further analysis. The samples were prepared with 2 μg of total RNA using the Enzo BioArray Single-Round RNA Amplification and Biotin Labeling System. Fifteen micrograms of the purified and fragmented aRNA were hybridized to 14 GeneChip RhG Arrays and 14 GeneChip HG-U133 Plus 2.0 Arrays. Image segmentation analysis and generation of DAT files was performed using Microarray Suite 5.0 (MAS5). Segmented images were normalized and $\log_2$ transformed using robust multiarray analysis separately for Rhesus and Human arrays (11). Robust multi-array analysis normalized $\log_2$ expression levels were utilized for all of our subsequent analyses.

Microarray data analysis

Microarrays were assessed for activity gene expression changes for each probe/gene (Figure 1). We tested for skew of the data using the following equation $(n / ((n-1)(n-2))) * \sum ((x_i - \bar{x}) / s)^3$. Data from RhG and HG arrays were fit using the model: $\log$ transformed gene expression value = $a + \beta(\text{activity})$. RhG and HG probe sets were matched on the basis of sequence identity using the Affymetrix HG-U133 Plus 2.0 to Rhesus Best Match Spreadsheet. Gene expression was considered correlated with spontaneous activity if (i) $P < 0.05$ for testing $H_0: \beta = 0$, using data for RhG and HG microarrays; and if (ii) the interquartile range > 0.263 (measuring the difference between the third and first quartiles was < 0.263) on the RhG and HG microarrays, which represented a 20% change in gene expression values). One-way hierarchical clustering of the replicating data was performed using GenePattern software (14) using $\log_2$ transformed robust multi-array analysis normalized expression levels utilizing row (gene) centering and Pearson correlation.

Quantitative real-time PCR Validation

We used High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) to synthesize cDNA from 500 ng of the same total RNA used for microarray analysis. Priming was performed with random hexamers. For each sample, amplified product differences were measured with three independent replicates using SYBR Green chemistry-based detection (12). Myelin basic protein was used as the endogenous reference gene. Primer sequences for myelin basic protein were 5' GGG TCT TCC TGG AGA TTT GG 3' and 5' GTG GTT TGG AAA CGA GGT TG 3', for AKT3 were 5' GCA AGG ATT GTA CAC GCT GA 3' and 5' GCA GCA TCT CTT CTC CCA AA 3', for CRBN were 5' CAA GCA TGA ACG GCA

GTC TA 3' and 5' CTT CAT GTC CCA TCA ATG ACA 3', for origin recognition complex subunit 4-like were 5' CAT GCT TTG AAA GAA CTC ATG CT 3' and 5' TGA TCT GCA GCA GTC CAT TT 3', and for PDK4 were 5' ATT GTC AGC TGC CTG GTC TT 3' and 5' GCC GTT ACT TTG GCA ATT CT 3'. The efficiency for each primer set was assessed prior to qPCR measurements, and a primer set was considered valid if its efficiency was >90%. The qPCR reactions were carried out on an ABI Prism 7300 thermal cycler (Applied Biosystems, Carlsbad, CA), quantified using ABI Prism 7300 SDS software, Carlsbad, CA (with the auto baseline and auto threshold detection options selected) and statistically analyzed using Pearson correlations.

Western Blots

Infrared imaging of immunoblots was used for assessing phosphorylation and total protein state of AKT (Li-Cor Biosciences, Lincoln, NE), and the total protein state of FOXO3. Samples were sonicated in a Triton-based lysis buffer (Cell Signaling, Danvers, MA) and subjected to standard sodium dodecyl sulfate-page immunoblotting procedures. Blots were then blocked in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween for 1 h prior to incubation overnight at 4 °C in primary antibody made in the same blocking solution. We used mouse anti-total AKT (Cell Signaling), rabbit antiphospho AKT (Ser473, Cell Signaling), mouse anti-FOXO3 (gift of Anne Brunet's laboratory, Stanford, CA), and mouse anti-β-actin (Sigma-Aldrich, St Louis, MO) to control for differences in protein loading. After overnight incubation with primary antibody, blots were washed in Tris-buffered saline-tween and incubated with goat secondary antibodies raised against the appropriate IgG fluorescing in the infrared range (700 and 800 nm, Li-Cor Biosciences). After three washes in Tris-buffered saline-tween, immunostained protein bands on the blots were visualized on an Odyssey Infrared Imager and quantified with Odyssey software (Li-Cor Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Experimental design. Motor cortex tissue was harvested from 14 female macaques with vastly different spontaneous activity levels. Their transcriptome was profiled using both Affymetrix Rhesus Macaque Genome microarrays (RhG) and Affymetrix Human Genome (HG) U133 2.0 Plus microarrays. We used a linear model to define gene expression associated with physical activity (gene value = $\alpha + \beta (\text{activity})$) for both microarrays separately. RhG and HG probes were cross matched based on sequence similarity and a transcript was considered associated with activity if both microarray models (**a**) were significant with $P < 0.05$ and (**b**) had an interquartile range (IQR) > 0.263 corresponding to a

20% gene expression change. Significant gene expression changes are reported in Supplementary Table S1 and clustered in Figure 2.

Figure 2.

Hierarchical clustering of gene expression associated with physical activity. One-way hierarchical clustering was performed on log₂-transformed expression levels of 98 differentially expressed genes using GenePattern. Monkeys are arranged by activity level in vertical columns. Monkey identities correspond to level of activity (e.g., Monkey ACT046378 had an average daily activity count of 46,378). Genes, denoted by Affymetrix Rhesus and Human Probes and NCBI gene symbols, were clustered horizontally. Each colored square represents a normalized gene expression value with color intensity proportional to magnitude of change (blue indicates lower expression and red indicated increased mRNA levels). **(a)** Clustering of Rhesus Genome Microarray Data. **(b)** Clustering of Human Genome Microarray Data. Note the progression of gene expression levels from monkeys with low activity to monkeys with high activity.

a

Correlation of Gene Expression with activity (qPCR)				
Activity (counts/day)	Activity (1/dCT)	CRBN (1/dCT)	ORC4L (1/dCT)	PDK4 (1/dCT)
46,478	0.357	0.249	0.226	0.174
73,961	0.371	0.247	0.230	0.182
118,067	0.383	0.253	0.244	0.190
123,780	0.403	0.251	0.230	0.203
129,822	0.499	0.282	0.296	0.366
134,284	0.488	0.303	0.302	0.337
143,509	0.398	0.260	0.229	0.184
183,932	0.573	0.287	0.280	0.335
191,266	0.542	0.302	0.270	0.272
240,431	0.498	0.284	0.279	0.220
271,736	0.479	0.280	0.300	0.326
485,187	0.633	0.334	0.382	0.481
Correlation with activity	0.795	0.804	0.867	0.754
Correlation Pvalue	0.0020	0.0016	0.0026	0.0046

b

Figure 3.

Validation of selected microarray-reported changes by qPCR. **(a)** Monkey activity levels were measured in counts/day, while AKT3, CRBN, ORC4L, and PDK4 relative expression levels are reported in threshold cycle difference relative to myelin basic protein (Δ CT). AKT3, CRBN, ORC4L, and PDK4 expressions were significantly positively correlated with activity. **(b)** Scatter plot showing qPCR expression (1/dCT) vs. activity levels (counts/day) for AKT3 (blue diamond), CRBN (red circle), ORC4L (black triangle), and PDK4 (green square).

Figure 4.

PhosphoAKT and FOXO3 protein levels are activity-dependent. **(a)** Scatter plot showing activity level (counts/day) vs. protein ratios for pAKT/actin (blue diamond) and for pAKT/tAKT (red square) with an insert of the original western blot data for four monkeys. We observed a significant correlation between activity level and pAKT expression ($P = 0.017$ vs. ACT and $P = 0.045$ vs. tAKT). **(b)** Scatter plot denotes activity level (counts/day) vs. protein ratios for FOXO3/actin (blue diamond) with an insert of the original Western blot data for four monkeys. The analysis revealed a significant correlation between activity level in the monkeys and FOXO3 expression of ($P = 0.012$). Note that physical activity level is correlated with both pAKT and FOXO3 protein levels.