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Exercise affects energy metabolism and neural plasticity-related proteins in the hippocampus as revealed by proteomic analysis

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Abstract

Studies were conducted to evaluate the effect of a brief voluntary exercise period on the expression pattern and post-translational modification of multiple protein classes in the rat hippocampus using proteomics. An analysis of 80 protein spots of relative high abundance on two-dimensional gels revealed that approximately 90% of the proteins identified were associated with energy metabolism and synaptic plasticity. Exercise up-regulated proteins involved in four aspects of energy metabolism, i.e. glycolysis, ATP synthesis, ATP transduction and glutamate turnover. Specifically, we found increases in fructose-bisphosphate aldolase C, phosphoglycerate kinase 1, mitochondrial ATP synthase, ubiquitous mitochondrial creatine kinase and glutamate dehydrogenase 1. Exercise also up-regulated specific synaptic-plasticity-related proteins, the cytoskeletal protein α-internexin and molecular chaperones (chaperonin-containing TCP-1, neuronal protein 22, heat shock 60-kDa protein 1 and heat shock protein 8). Western blot was used to confirm the direction and magnitude of change in ubiquitous mitochondrial creatine kinase, an enzyme essential for transducing mitochondrial-derived ATP to sites of high-energy demand such as the synapse. Protein phosphorylation visualized by Pro-Q Diamond fluorescent staining showed that neurofilament light polypeptide, glial fibrillary acidic protein, heat shock protein 8 and transcriptional activator protein pur-alpha were more intensely phosphorylated with exercise as compared with sedentary control levels. Our results, together with the fact that most of the proteins that we found to be up-regulated have been implicated in cognitive function, support a mechanism by which exercise uses processes of energy metabolism and synaptic plasticity to promote brain health.

Introduction

The beneficial effects of exercise on the function of the brain and spinal cord are becoming well recognized. A plethora of studies have provided evidence that exercise enhances cognitive function in both humans and animals (Fordyce & Wehner, 1993; Kramer *et al.*, 1999; Laurin *et al.*, 2001). Substantial progress has been made in the last few years in defining the mechanisms underlying the action of exercise on the central nervous system. However, given the complexity of biological functions activated by exercise, there remain major gaps in our knowledge. In particular, it is not well understood how exercise orchestrates the action of multiple factors at the protein level. The development of proteomic technology to scrutinize multiple proteins in the same preparation offers the unique opportunity for studies of complex biological functions, which inherently involve a large number and network of proteins. Proteomics enables us to evaluate protein complexes, signalling pathways and protein changes (Grant & Blackstock, 2001; Phizicky *et al.*, 2003; Tyers & Mann, 2003) to further our understanding of the protein interactions involved in the cellular machinery of exercise.

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The goal of the present study was to use a proteomic approach to examine the molecular diversity in the expression pattern and post-translational modifications of protein classes in the hippocampus of rats exposed to exercise. We chose to examine the hippocampus as it is a critical brain region involved in supporting learning and memory processes. Our results showed that exercise up-regulates multiple proteins that have a defined role in energy metabolism, comprising enzymes involved in glucose catabolism, ATP synthesis and glutamate turnover. Moreover, our results showed that exercise increases specific classes of proteins related to synaptic plasticity, i.e. proteins involved in cytoskeletal structure and protein-folding dynamics. Studies have shown that exercise influences molecular systems associated with synaptic function and plasticity (Tong *et al.*, 2001; Molteni *et al.*, 2002; Vaynman *et al.*, 2003) that may ultimately serve higher-order functions such as learning and memory. The fact that brain energy expenditure accounts for about 80% of total body energy consumption, when the brain itself constitutes only about 2% of body weight, emphasizes the importance of energy metabolism in supporting neuronal function and plasticity. Although exercise is intrinsically related to energy metabolism (Maddaiah, 1984; Constable *et al.*, 1987; Westerterp & Plasqui, 2004), the possibility that exercise can affect energy-related proteins has been poorly studied in the brain. Proteomics has enabled us to show that exercise up-regulates maps of proteins related to neurometabolism (Lubec *et al.*, 1999). A

unifying theme in our findings is that all proteins affected by exercise have been implicated in cognitive function. These results seem to indicate that the impact of exercise on the brain is achieved by coordination with mechanisms that modulate energy metabolism and synaptic plasticity.

Materials and methods

Exercise paradigm

Adult male Sprague-Dawley rats (approximately 220 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were randomly assigned to two groups, exercise and sedentary ($n = 5$ per group), and individually housed in standard polyethylene cages in a 12-h light/dark cycle at 20–22 °C with food and water *ad libitum*. The exercise rats were given access to a wheel (diameter 31.8 cm, width 10 cm) that freely rotated against a resistance of 100 g and was connected to a computer system (VITALVIEWER DATA ACQUISITION SYSTEM, Minimitter Company, Inc., Sunriver, OR, USA), which monitored their revolutions. We chose to use a voluntary exercise model as our paradigm because it simulates aspects of human behaviour by enabling animals to choose how much to run. The sedentary control rats were confined to the cages with no access to a running wheel. Exercise rats were exposed to five consecutive nights of the running wheel. On the morning immediately following the last night of running, rats were killed by decapitation. Hippocampi were quickly removed, frozen on dry ice and stored at −70 °C until used. All animal protocols were approved by the UCLA Animal Research Committee and followed the guidelines of the American Physiological Society concerning animal care.

Materials

Immobiline DryStrips, CHAPS, acrylamide/Bis solution, dithiothreitol, TEMED, ammonium persulphate and the Reagent Compatible and Detergent Compatible (RCDC) protein assay kit were from Bio-Rad (Hercules, CA, USA). Pepstatin A, leupepsin, α-cyano-4-hydroxycinnamic acid and ProteoProfile™ PTM marker were from Sigma (St Louis, MO, USA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). The Pro-Q® Diamond phosphoprotein gel stain kit and SYPRO® Ruby protein gel stain kit were from Molecular Probes (Eugene, OR, USA).

Sample preparation

The rat hippocampus was homogenized in the lysis buffer containing 8 M urea, 4% w/v CHAPS and 0.8% Biolytes 100 mM dithiothreitol. Protease inhibitors, 10 μg/mL aprotinin, 1 μg/mL leupeptin and 1 mM phenylmethylsulphonyl fluoride, were contained in the lysis buffer. The protein concentration was determined with the RCDC method with bovine serum albumin as the protein standard.

Two-dimensional gel electrophoresis

The two-dimensional gel electrophoresis gels for the exercise and sedentary groups were run in parallel, such that each rat had a corresponding gel. The two-dimensional gel electrophoresis gels for isoelectric focusing were run using the following presettings (24-cm immobilized ph gradient strips, pH 3–10, 500 mg protein loaded): 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V (gradient) for 0.5 h, maximizing at 8000 V when the total gel running sequence (200–8000 V) reached 30 kV.h (kV × hours). For the second dimension, sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels for the exercise and sedentary groups were run in parallel (260 × 200 × 1.5 mm, 12.5% total monomer, 2.6% crosslinker). No samples were pooled.

Gel staining

The two-dimensional gels were first stained with Pro-Q Diamond dye for visualizing phosphoprotein followed by SYPRO Ruby for visualizing total proteins. Gel imaging was performed on a Molecular Imager FX (Bio-Rad) using excitation and emission wavelengths for both protein dyes. Pro-Q Diamond has an excitation maximum at ∼555 nm and emission at ∼580 nm, and SYPRO Ruby has two excitation maxima, one at ∼280 nm and one at ∼450 nm, and the emission maximum is near 610 nm. ProteoProfile™ PTM marker (Sigma) was used as the positive phosphoprotein standard. Imaging analysis and comparison (between sedentary and exercise groups) were fulfilled with PDQUEST software (Bio-Rad).

In-gel digestion

Selected protein spots in two-dimensional gels were automatically excised by ProteomeWorks Spot Cutter (Bio-Rad). Gel plugs were dried on a Savant speedvac evaporator (Thermo Electron Corporation, Waltham, MA), rehydrated with 5 μL of 10 ng/μL trypsin solution in 50 mM NH_4HCO_3 and incubated overnight at 37 °C. The digested peptide fragments were extracted twice with 50 μL of 5% trifluoroacetic acid in 50% acetonitrile.

Mass spectrometry

Peptide mass spectra were recorded in positive ion mode on a Voyager DE-STR MALDI time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA). Mass spectra were obtained by averaging 500 individual laser shots. Two trypsin autohydrolytic peptide isotopic peaks (m/z 842.5094 and 2211.1046) were used for internal calibration standards. When m/z 2211.1046 failed to be observed, an external standard of 0.65 pmol of the synthetic peptide A19L ($C_9H_{150}N_{25}O_{29}$) was mixed with samples, thereby employing m/z 2025.1133 as an internal calibration standard. Selected proteins were identified by MALDI time-of-flight mass spectrometry. Methodologically, four to five matched peptide fragments with mass accuracy < 50 parts per million (p.p.m.) can unambiguously identify a known protein from the database (Berndt *et al.*, 1999). Here, we used more stringent criteria for accepting a database hit. First, due to double internal calibration, the mean mass accuracy of matched proteins used was 11 p.p.m., far < 50 p.p.m.. In addition, most of the identified proteins had more than five matched masses (on average 10 masses matched for each protein) and the average sequence coverage for all identified proteins was 30% (Table 1 shows the average sequence coverage for each protein spot). We also took the relative mass intensity into consideration; for example, for the most intensive masses the three to five strongest signals had to be covered in the matched protein. Such an additional criterion efficiently distinguished the target protein from the interference background. In most cases, the experimental molecular weights and isoelectric points (pIs) matched protein theoretical data (Table 1). To confirm the reliability of the MALDI time-of-flight mass spectrometry, a second method, tandem mass spectrometric analysis, was used to identify two random proteins, glutamate dehydrogenase (GDH)1 and ATP synthase. Tandem mass spectrometric analysis was performed using a QSTAR XL QqTOF MS (Applied Biosystems/MDS Sciex, Applied Biosystems) with the NanoSpray™ ion sources.

TABLE 1. Hippocampal proteins identified from two-dimensional gel by MALDI time-of-flight mass spectrometry

Spots	Protein name	Swiss-protein entry	Mw/pI*, Experimental	Mw/pI*, Theoretical	Sequence coverage	Matched experimental masses	Mass error (mean, p.p.m.)
1	Aconitase, mitochondrial	ACON_RAT	85/7.9	85/7.9	24%	935.5, 985.51, 1067.57, 1170.69, 1463.75, 1500.77, 1581.72, 1601.8, 1667.77, 1861.79, 1868.91, 2129.16, 2158.03, 2780.44	8
2	Actin beta	ACTB_RAT	45/5.6	42/5.23	36%	795.47, 976.45, 1132.53, 1198.71, 1515.77, 1790.92, 1954.04, 2231.05, 2359.13,3199.57	8
3	Actin beta	ACTB_RAT	40/5.3	42/5.3	39%	795.46, 923.57, 976.46, 1132.53, 1198.7, 1515.76, 1516.72, 1790.9, 1954.06, 2231.07, 3199.7	9
4	Actin beta	ACTB_RAT	35/5.6	42/5.3	39%	795.47, 976.45, 1132.53, 1198.71, 1515.76, 1516.73, 1790.91, 1954.05, 2231.06, 3199.6	6
5	Aldo-keto reductase	AK1A1_RAT	38/7.0	36/6.8	33%	897.56, 928.5, 1285.67, 1375.76, 1505.65, 1551.79, 1928.02, 2098.1, 2202.07	14
6	Aldolase A,	ALDOA_RAT	36/8.4	39/8.7	30%	1044.58, 1342.73, 1646.83, 1668.83, 1824.94, 2123.12, 2139.11, 2272.18, 3039.54	11
7	Aldolase A	ALDOA_RAT	35/8.9	39/8.7	37%	1107.59, 1342.71, 1646.81, 1646.81, 1668.82, 1808.93, 1808.93, 1824.93, 2088.1, 2091.12, 2258.04, 2272.16	4
8	Aldolase C	ALDOC_RAT	40/6.4	39/6.7	44%	1018.51, 1050.54, 1173.67, 1421.71, 1644.83, 1652.94, 1667.82, 1810.9, 1811.9, 2299.19, 3067.59	9
9	Aldolase C	ALDOC_RAT	40/6.9	39/6.7	23%	1644.87, 1652.95, 1667.81, 2299.18, 3067.58	10
10	Aldolase C	ALDOC_RAT	39/7.4	39/6.7	38%	1018.51, 1173.65, 1421.72, 1488.8, 1644.83, 1652.94, 1667.82, 2100.94, 2299.18, 3067.56	11
11	ATP synthase alpha chain	ATPA_RAT	48/8.7	59/9.3	33%	892.49, 1026.6, 1438.84, 1483.81, 1553.75, 1575.82, 1610.87, 1683.76, 2325.15, 2338.18, 2338.18, 2369.26	7
12	ATP synthase alpha chain	ATPA_RAT	49/8.7	59/9.3 55/8.3	29%	892.51, 1000.6, 1026.6, 1438.86, 1553.76, 1575.8, 1610.89, 1911.02, 2325.14, 2338.19, 2369.24	11
13	ATP synthase alpha chain	ATPA_RAT	49/8.6	55/8.3	31%	1000.58, 1026.58, 1438.85, 1483.84, 1553.74, 1575.8, 1610.88, 1683.8, 2325.14, 2338.18, 2369.25	7
14	ATP synthase beta chain	ATPB_RAT	50/5.6	56/5.2	40%	1038.6, 1401.7, 1406.69, 1435.76, 1439.79, 1617.8, 1650.93, 1831.86, 1919.11, 1921.99, 1988.03, 3842.95	5
15	ATP synthase beta subunit	ATPB_RAT	49/5.3	51/4.9	52%	975.57, 1038.6, 1401.69, 1406.7, 1435.77, 1439.8, 1617.8, 1650.94, 1831.86, 1919.13, 1922.00, 1988.06, 2038.98, 2298.07, 2334.16,2691.39, 3714.9, 3714.9, 3843.03 (IGLFGGAGVGK;IPVGPETLgr;VVDLLAPYAK)†	10
16	Chaperonin, (CCT) beta subunit	TCPQ_MOUSE	49/5.8	59/5.4	22%	1130.56£1330.68£1582.94£1956.1£2097.12£2288.18£2363.18	9
17	Cofilin 1	COF1_RAT	16/8.5	18/8.5	36%	769.34, 1309.68, 1337.62, 1790.86	11
18	Creatine kinase, mitochondrial/(uMtCK)	KCRU_RAT	42/8.1	47/8.7	28%	873.39, 1071.6, 1270.54, 1420.74, 1548.83, 1576.84, 1645.82, 1677.83, 1734.96, 2463.3, 2960.33	21
19	Creatine kinase, brain B chain	KCRB_RAT	45/5.7	43/5.3	36%	1230.56, 1303.73, 1557.79, 1602.85, 1671.86, 1864.95, 1964.95, 2121.03, 2471.11, 2518.19	7
20	Cytochrome c oxidase, subunit Va	COX5A_RAT	16/4.8	12/5.0	38%	992.56, 1148.6, 2053.05, 3428.8	20
21	acetyltransferase	ODP2_RAT	68/5.7	59/5.7	10%	884.53, 1188.66, 1754.94, 1880.07	7
22	Dihydropyrimidinase-related protein-2	DPYL2_RAT	65/5.8	62/5.9	30%	766.45, 795.44, 908.49, 1015.55, 1140.61, 1310.68, 1682.89, 1792.83, 1911.03, 2169.08, 2182.1, 2377.18, 2900.51	7
23	Enolase 2, gamma, neuron specific, NSE	ENOG_RAT	45/5.3	47/5.0	22%	1130.59, 1620.73, 1620.73, 1804.94, 1855.89, 1970.97, 2102.09	12
24	Enolase 2, gamma, neuron specific, NSE	ENOG_RAT	44/5.3	47/5.0	34%	776.43, 848.42, 1174.58, 1599.73, 1620.71, 1804.95, 1855.89, 1970.93, 2102.1, 2220.19, 2353.1	14
25	Enolase 2, gamma, neuron specific, NSE	ENOG_RAT	45/5.1	47/5.0	29%	776.41, 840.37, 848.41, 1130.62, 1174.56, 1620.72, 1804.95, 1855.91, 1970.94, 2102.06	14
26	Enolase , non-neural	ENOA_RAT	48/5.8	47/6.2	20%	1439.76, 1804.95, 1928.95, 1960.92, 2208.01	6
27	Enolase, non-neural,	ENOA_RAT	47/5.8	47/6.2	30%	704.41, 766.36, 806.46, 1143.65, 1439.74, 1557.74, 1804.94, 1928.93, 2047.04, 2207.98	13
28	Enolase, non-neural, NNE1	ENOA_RAT	47/5.8	47/6.2	24%	704.39, 766.36, 806.45, 1439.73, 1804.95, 1928.96, 2047.04, 2208.01	10
29	Ferritin light chain	FRIL1_RAT	28/7.5	35/6.4	15%	1173.59, 1213.62, 1309.62, 1586.80	14

TABLE 1. (Continued)

Spots	Protein name	Swiss-protein entry	Mw/pI*, Experimental	Mw/pI*, Theoretical	Sequence coverage	Matched experimental masses	Mass error (mean, p.p.m.)
30	Glial fibrillary acidic protein delta (GFAP)	Q9Z2S0_RAT	49/5.6	49/5.7	61%	715.41, 793.47, 898.49, 905.44, 959.5, 988.52, 1039.57, 1060.51, 1094.56, 1098.64, 1177.63, 1224.6, 1245.66, 1277.72, 1291.68, 1387.77, 1409.74, 1499.81, 1546.8, 1619.89, 1629.95, 1664.8, 1795.93, 1871.99,3076.54	10
31	Glial fibrillary acidic protein delta (GFAP)	Q9Z2S0_RAT	59/5.7	49/5.7	41%	959.49, 988.51, 1094.55, 1098.63, 1177.64, 1224.59, 1245.65, 1277.72, 1291.68, 1387.77, 1409.74, 1499.77, 1546.8, 1619.89, 1629.94, 1872.02, 1872.02, 3076.55	9
32	GAPDH	G3P_RAT	36/7.9	36/8.4	13%	1556.82, 1627.97, 1779.82	10
33	GAPDH	G3P_RAT	36/8.7	36/8.7	27%	665.36, 805.43, 811.39, 1179.61, 1556.83, 1627.96, 1779.82, 2611.33	11
34	GAPDH	G3P_RAT	36/8.5	36/8.7	22%	811.41, 1556.83, 1627.96, 1779.86, 2611.32	14
35	GAPDH	G3P_RAT	36/8.2	36/8.4	22%	811.39, 1556.82, 1627.98, 1779.81, 2611.32	14
36	GAPDH	G3P_RAT	36/7.9	36/8.7	20%	1556.87, 1627.96, 1779.8, 2611.34	12
37	Guanine deaminase	GUAD_RAT	46/5.6	51/5.5	25%	998.52, 1058.63, 1173.63, 1371.8, 1544.75, 1700.87, 2074.99, 2087.07, 2147.13,2511.26	14
38	H+-ATP synthase atpase, V1 subunit A	Q6IRF8_RAT	74/5.6	68/5.4	32%	885.44,1145.61, 1230.64, 1262.63, 1308.73, 1316.74, 1487.73, 1731.82, 1731.82, 1749.88, 1797.81, 1883.78, 2042, 2086.05, 2143.06, 2186.12, 2681.26	16
39	H+-ATP synthase,	AT5G2_RAT	22/5.8	19/6.2	32%	1177.55, 1338.64, 1417.72, 1980.03	9
40	HSP 60kD (HSP60)	CH60_RAT	58/5.7	61/5.9	17%	1684.89, 1905.07, 1938.9, 2033.15, 2365.32, 2560.25	8
41	HSP 70Kd	Q9DC41_MOUSE	79/5.6	72/5.1	36%	986.53,997.51, 1074.56, 1191.64,1210.6,1224.59, 1228.64,1233.62, 1316.65, 1329.62, 1460.78, 1528.75, 1566.79, 1588.88,1590.79, 1590.79, 1604.83, 1816.01, 1887.98, 1934.04, 1999.11, 2016.07, 2149.02, 2178.02	11
42	Heat shock protein 8 (Hsc71)	HSP7C_RAT	73/5.6	71/5.4	22%	1081.55, 1199.68, 1228.63, 1253.63, 1480.76, 1487.73, 1691.74, 1982.01, 2774.3, 2997.44	9
43	Hsp70/Hsp90-organizing protein	STIP1_RAT	64/6.3	63/6.4	16%	1004.54, 1065.56, 1116.64, 1132.64, 1242.66, 1298.64, 1455.74, 1488.8,1812.98	10
44	Internexin alpha	AINX_RAT	63/5.6	56/5.2	24%	1056.62, 1133.57, 1518.87, 1544.79, 2143.08, 2175.06, 2527.39	12
45	Isovaleryl-coa dehydrogenase	IVD_RAT	43/8.6	46/8.5	14%	840.41, 1012.54, 1280.68, 1467.76, 2301.18	13
46	LDH, B isoform	LDHB_RAT	38/5.7	36/5.7	24%	742.46, 913.59, 957.6, 1186.64,1248.6, 1629.84, 2312.15	6
47	Memory related protein-2 (GDH-1)	DHE3_RAT	51/7.2	56/6.7	48%	963.54, 1016.45, 1059.55, 1196.68, 1425.64, 1507.72, 1723.89, 1764.88, 1894.09, 1931.91, 1936.92, 2242.17 (YNLGLDLR;ALASLMTYK;YSTDVSV-DEVK)†	6
48	Myelin basic protein	MBP_RAT	17/8.5	21/11.2	45%	726.38, 728.33, 1110.53, 1339.7, 1352.61, 1460.71,1800.85	11
49	Neurofilament, light polypeptide	NFL_RAT	69/4.8	61/4.6	20%	869.47, 1021.54, 1021.54, 1072.56, 1154.73, 1281.63, 1723.8, 1747.88, 2295.13	14
50	PGP9.5	UCHL1_RAT	26/5.5	25/5.1	37%	886.47, 1863.83, 1983.95, 2230.04, 2519.33	25
51	PGP9.5	UCHL1_RAT	26/5.4	25/5.1	36%	886.5, 980.51, 1983.91, 2230.05, 2519.26, 2976.51	10
52	Neuronal protein 22 (NP22)	Q8VHH3_RAT	22/6.6	23/6.8	31%	965.46, 1015.47, 1121.58, 1267.67, 1665.93, 2358.21	11
53	Nucleoside diphos-phate kinase B	NDKB_RAT	17/6.9	17/6.9	32%	1051.5, 1175.67, 1344.77, 1801.88	9
54	3-Oxoacid coA transferase	Q5XIJ9_RAT	51/7.4	56/7.1	14%	776.42, 1647.76, 2159.15, 2217.15, 2407.11	14
55	Peroxiredoxin 2	PRDX2_RAT	22/5.5	22/5.3	28%	789.42, 937.45, 1108.59, 1211.69, 1706.91, 1835.07, 2699.36, 2827.39	15
56	Phosphatidylethanol-amine binding protein	PEBP2_MOUSE	22/5.6	21/5.5	29%	912.49, 1560.83, 1688.93, 1758.85, 1963.94	9
57	Phosphoglyce-rate kinase 1 (PGK-1)	PGK1_RAT	39/8.7	44/8.3	23%	1101.55, 1634.81, 1769.02, 2067.07, 2772.35	12
58	Phosphoglycerate mutase B chain	Q6P0K6_RAT	32/6.3	29/6.7	32%	1150.69, 1312.63, 2131.12, 2425.19, 2433.05	15

TABLE 1. (Continued)

Spots	Protein name	Swiss-protein entry	Mw/pI*, Experimental	Mw/pI*, Theoretical	Sequence coverage	Matched experimental masses	Mass error (mean, p.p.m.)
59	Prohibitin	PHB_RAT	32/5.7	30/5.6	48%	1058.54, 1149.6, 1185.67, 1396.85, 1460.66, 1606.86, 1998.1, 2119.12, 2371.28	9
60	Proteindisulfide-isomerase A3	PDIA3_RAT	59/5.7	57/6.0	15%	1188.52, 1191.61, 1397.72, 1529.79, 1587.83, 2302.24	15
61	Pyruvate kinase	Q6P7S0_RAT	52/7.0	53/8.4	13%	990.56, 1359.71, 1473.82, 1875.9, 2435.3	12
62	Serum albumin precursor	ALBU_RAT	71/5.7	69/6.1	26%	1266.62, 1439.8, 1455.8, 1465.8, 1479.8, 1609.81, 1662.86, 1882.94, 1948.93, 2060.04, 2139.01	10
63	Serum albumin precursor	ALBU_RAT	71/5.7	69/6.1	34%	1134.47,1149.58, 1266.6, 1299.71, 1439.78, 1455.8, 1465.78, 1479.81, 1563.74, 1609.8, 1662.86, 1684.82, 1714.8, 1882.94, 1948.95, 2060.04, 2139.00	13
64	Synapsin II	SYN2_RAT	55/6.3	52/8.4	8%	1249.70, 1567.75, 1677.83	22
65	Synaptosomal-assoc-iatedprotein 25 kda	SNP25_RAT	25/4.7	21/4.7	43%	1669.81, 1669.81, 1676.81, 1776.74, 1822.91, 2175.99	10
66	Transcriptional activator	PUR_MOUSE	40/5.9	35/6.1	37%	750.43, 788.37, 1060.5, 1078.61, 1216.61, 1368.7, 2311.25, 2889.39, 3270.53	9
67	Tubulin alpha 1	TBA1_RAT	57/4.7	50/4.9	22%	1023.45, 1085.64, 1265.55, 1410.79,1487.9,1701.94, 1718.9, 1756.96	10
68	Tubulin alpha 1	TBA1_RAT	58/4.9	50/4.9	14%	1487.84 1593.87 1701.91 1718.86 2409.22	10
69	Tubulin alpha 1	Q52L87_MOUSE	57/5.0	50/4.9	18%	1085.62, 1410.76, 1487.88, 1701.92, 1718.9, 1756.96, 2409.19	7
70	Tubulin alpha 6	Q52L87_MOUSE	57/5.8	63/5.9	14%	1487.9, 1701.91, 1718.88, 2409.22	6
71	Tubulin beta 15	TBB1_RAT	50/4.7	48/4.7	29%	1039.6, 1077.52, 1130.6, 1159.62, 1245.58, 1258.72, 1287.72, 1401.68, 1401.68, 1631.83, 1636.82, 1659.89,1959.01, 2798.37	9
72	Tubulin beta 15	TBB1_RAT	50/4.5	48/4.7	41%	1039.59, 1077.52, 1130.59, 1159.61, 1245.58, 1258.71, 1287.69, 1401.78, 1631.82, 1636.82, 1659.82, 1696.79, 1822.86, 1959.01, 2724.31, 2798.39	19
73	Tubulin beta 15	TBB1_RAT	58/5.5	50/4.8	41%	1028.52, 1039.6, 1077.53, 1130.6, 1159.62, 1245.59, 1258.69, 1287.71, 1620.84, 1631.83, 1636.83, 1958.99, 2724.33, 2798.37, 3102.43	4
74	Tubulin beta 15	TBB1_RAT	58/5.6	50/4.8	40%	738.35, 1028.53, 1039.6, 1077.52, 1130.6, 1159.61, 1245.58, 1258.69, 1287.72, 1631.84, 1636.81, 1822.84, 1958.98, 2724.33, 2798.34	9
75	Tubulin beta 15	TBB1-RAT	58/5.7	50/4.8	52%	534.3, 738.36, 1028.52, 1053.61, 1077.52, 1130.6, 1143.63, 1159.62, 1229.61, 1245.59, 1258.7, 1287.71, 1620.84, 1631.83, 1636.82, 1696.81, 1822.92, 1873.94, 1958.99, 2724.32, 3102.42	6
76	Tubulin beta 15	TBB1_RAT	50/4.5	48/4.7	23%	1077.52, 1130.6, 1159.61, 1258.74, 1287.71, 1631.81, 1636.81, 1959.00 , 2798.30	15
77	Tubulin beta 15	TBB1_RAT	35/5.6	50/4.5	35%	534.31, 738.34, 1077.53, 1130.61, 1159.62, 1258.71, 1287.72, 1355.67, 1631.82, 1636.81, 1723.9, 1822.93, 1958.99, 2798.35	10
78	Ubiquitin	UBIQ_RAT	11/6.6	9/6.6	47%	1039.51, 1067.62, 1523.78, 1787.92, 2130.16	3
79	Valosin-containing protein	TERA_RAT	95/5.6	89/5.1	21%	1067.53, 1329.72, 1645.83, 1823.91, 1823.91, 2185.94, 2498.33, 2518.41, 3672.84	14
80	Voltage-dependent anion channel 1	VDAC1_RAT	31/8.2	32/8.3	33%	1946.03, 2103.17, 2128.05, 2176.06, 2600.18	8

A stringent criterion for protein identification was used, employing a mean mass accuracy of 11 p.p.m. with more than five matched masses for most of the identified proteins. *Molecular weight/isoelectric point. †Matched peptide fragment sequences from tandem mass spectrometric analysis.

Database search

Protein identification with peptide mass data was accomplished using the ProFound searching tool against the latest version of non-redundant database (NCBInr) (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe?FORM=1). Interference mass signals from matrix, keratins and trypsin were manually picked out before database searching (Ding *et al.*, 2003). Searching parameter presettings were as follows: maximum cleavage missing, 1; mass tolerance, ±0.1 Da; taxonomic category, *Rattus*; cysteine and methionine residues partially alkylated and oxidized, respectively. The ProFound search engine was used to calculate the Bayesian probability of each candidate sequence being the protein analysed by comparison of search results against the estimated random match population (Zhang & Chait, 1995.). *Z*-scores were obtained based on the probability value of each protein candidate. *Z*-scores > 1.65 were considered significant ($P < 0.05$). Only proteins with an expression fold change ≥1.6 (previously established in Ding *et al.* 2006) with a *Z*-score of > 1.65 were considered as biological differences differentially expressed by exercise.

Western blot

Protein changes of creatine kinases in the hippocampus were checked by western blot, using the tissue from the same animals used for proteomic analysis ($n = 5$ animals per group). Briefly, equal amounts of protein samples (25 μg) were separated on a 12.5% polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane. Non-specific binding sites were blocked for 1 h at room temperature (25 °C) with 5% non-fat milk in Tris-buffered saline with tween buffer, pH 7.5, and then incubated overnight at 4 °C with the primary antibody of either brain-type creatine kinase (CKBB) (1 : 1000, rabbit IgG, Biogenesis) or ubiquitous mitochondrial creatine kinase (uMtCK) (1 : 1000, goat IgG, Santa Cruz Biotechnology) followed by 1 h incubation at room temperature with secondary anti-rabbit (or goat) horseradish peroxidase conjugate (1 : 10 000). As previously described, actin (primary antibody 1 : 2000; Santa Cruz Biotechnology, followed by the secondary anti-goat horseradish peroxidase conjugate) was used as the standard internal control (Molteni *et al.*, 2004; Vaynman *et al.*, 2006a, b) in the CKBB and uMtCK western blots. Immuno-complexes were visualized by chemiluminescence using the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Statistical differences were considered significant when $P < 0.05$. We used the mean value of the sedentary control group to calculate the percentage for each animal for the sedentary and exercise groups. A *t*-test was used for between-group comparisons. Values represent the mean ± SEM.

Results

Representative two-dimensional maps of overall protein expression from the hippocampus of sedentary and exercise rats are shown in Fig. 1. The majority of proteins were distributed on the left and central regions of the map in the Cartesian coordinate system. What this means is that the major proteins displayed here are acidic and neutral proteins. This is consistent with the fact that nervous system proteins are mainly composed of acidic and neutral proteins (Yang *et al.*, 2004; Witzmann *et al.*, 2005). In terms of protein size, the majority of proteins were between 30 and 100 kDa. Differentially displayed proteins induced by exercise were found by map matching. Enlarged areas from boxes in Fig. 1A and B are shown in the panels underneath (Fig. 1C and D) and demonstrate representative protein expression changes.

A total of 80 protein spots of relatively high abundance were identified by peptide mass mapping (Table 1). Several proteins had

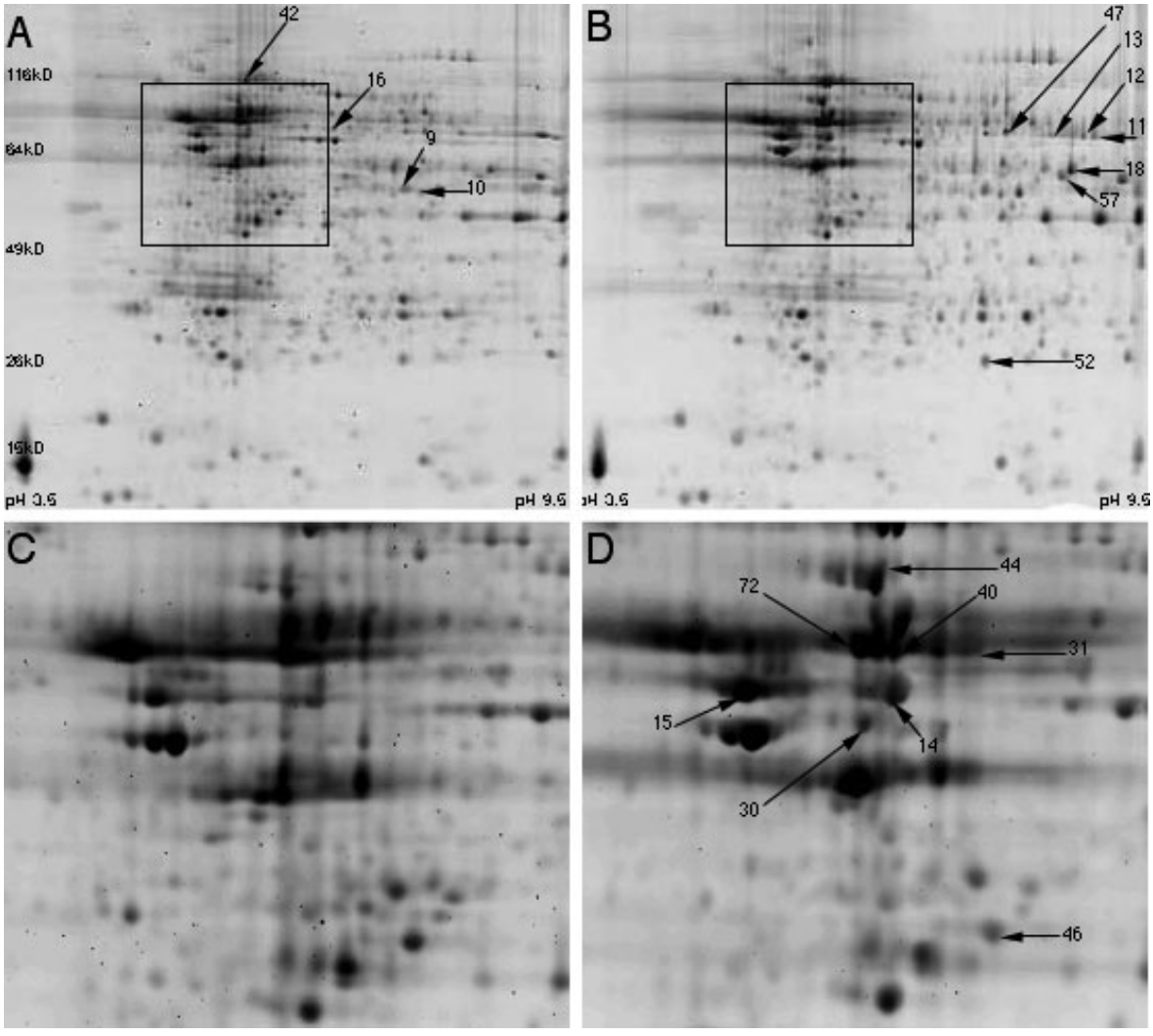

FIG. 1. Representative two-dimensional gel electrophoresis maps of the hippocampus from sedentary (A) and exercise (B) rats. The boxes in A and B represent the areas enlarged in C and D showing the position of protein spots. All protein spots differentially expressed by exercise in Tables 2 and 3 have been labelled to be consistent with the number designation set out in Table 1.

TABLE 2. Protein increases related to energy metabolism in the adult rat hippocampus after exercise

Protein name	Spots	Total protein (fold change)	Function	*Z*-score
Energy generation				
Fructose-bisphosphate aldolase C	9	2.8	Brain isoform, glycolysis	2.43
	10	1.7		2.43
Phosphoglycerate kinase 1 (PGK-1)	57	1.9	glycolysis	2.16
Lactate dehydrogenase B (LDH)	46	1.8*	Turnover of pyruvate to lactose	2.25
ATP synthase alpha chain, mitochondrial precursor	11	1.9	Mitochondrial, F1 unit	2.43
	12	3.0		2.37
	13	3.0		2.43
ATP synthase beta chain, mitochondrial precursor	14	1.9	Mitochondrial, F1 unit	2.43
	15	1.8		2.43
Energy transduction				
Ubiquitous mitochondrial creatine kinase (uMtCK)	18	1.7	Mitochondrial	2.30
Glutamate turnover				
Glutamate dehydrogenase-1 (GDH-1, memory related protein 2)	47	1.7	Glutamate breakdown	2.14

Proteins have been organized according to their functional and sequential roles in energy metabolism, to comprise the categories of energy generation, energy transduction and glutamate turnover. A protein fold change of ≥ 1.6 and a *Z*-score of > 1.65 were considered to reflect a significant effect of exercise on protein expression, i.e. the proteins were considered to be differentially expressed by exercise. *LDH protein decreased.

multiple protein spots, possibly due to post-translational modifications. Protein expression changes of two creatine kinases, i.e. cytosolic CKBB and mitochondrial uMtCK, were confirmed by western blot (Fig. 3).

The Pro-Q Diamond phosphoprotein gel stain provided a method for selectively staining phospho-proteins in polyacrylamide gels (Martin *et al.*, 2003). This fluorescent stain allowed the direct in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues. Representative Pro-Q Diamond-stained spots are shown in Fig. 3. Phosphorylation intensity fold changes and possible phosphate sites are listed in Table 4, from which the phosphorylated proteins fall into the category of plasticity-related proteins. We found that the phosphorylated proteins glial fibrillary acidic protein (GFAP), neurofilament light polypeptide (NFL), transcriptional activator protein pur-alpha (TAPP), β-tubulin and heat shock protein 8 exhibited changed fold increases with exercise (Table 4). For illustrative purposes, all exercise-modified proteins are grouped in Tables 2–4 according to their functions and are summarized below.

TABLE 3. Differential increases of proteins involved in synaptic plasticity in the hippocampus after 5 days of voluntary wheel running as revealed by proteomic analysis

Protein name	Spots	Total protein (fold change)	*Z*-score
Cytoskeletal proteins			
Beta tubulin	72	1.9	1.89
Glial fibrillary acidic protein delta (GFAP)	30	1.7	2.43
	31	1.7	2.43
α-internexin	44	2.0	2.03
Chaperones			
Heat shock protein 8 (Hsc71)	42	3.4	1.72
Heat shock 60Kd protein 1 (HSP60)	40	2.0	2.43
Chaperonin containing TCP-1 beta subunit (CCT)	16	1.9	2.38
Neuronal protein 22 (NP22)	52	2.2	2.43

Proteins have been organized according to their functional roles in synaptic plasticity, comprising the categories of cytoskeletal proteins and chaperones. A protein fold change of ≥ 1.6 and a *Z*-score of > 1.65 were considered to reflect a significant effect of exercise on protein expression, i.e. the proteins were considered to be differentially expressed by exercise. TCP-1, t-complex peptide 1.

Proteins involved in energy metabolism

Enzymes involved in glucose catabolism

We found that many enzymes pertaining to glycolysis were affected by exercise (Table 2). Exercise up-regulated the brain isoform of aldolase C and phosphoglycerate kinase 1 (PGK-1). We found two protein spots for aldolase C with change fold increases of 1.7 and 2.8 (Table 2). PGK-1 exhibited a fold change increase of 1.9 (Table 2). In contrast, enzymes involved in the subsequent steps of energy metabolism related to the tricarboxylic acid cycle, such as dihydrolipoyllysine-residue acetyltransferase and aconitase, remained unchanged. Mitochondrial isovaleryl-coA dehydrogenase, an enzyme involved in amino-acid catabolism, remained unchanged during exercise. We found that the memory-related GDH-1, an enzyme facilitating the turnover of the excitatory neurotransmitter glutamate to 2-oxoglutarate was differentially expressed by exercise, exhibiting a protein fold change of 1.7 (Table 2).

TABLE 4. Differential increases in protein phosphorylation in the hippocampus after 5 days of wheel running

Protein name	Protein (fold change)		Possible phosphosites*
	Phospho-protein	Total protein	
Beta tubulin	2.0	1.9	GIDPTGS**Y**^{36}HGDSDLQ
Neurofilament light polypeptide	2.7	1.1	SSLSVRR**S**^{55}YSSSSGS
			SSGSLMP**S**^{66}LENLDLS
			EAKDEPP**S**^{472}EGEAEEE
Glial fibrillary acidic protein delta	1.7	1.7	MERRRI**T**^{7}SARRSYA
			MERRRIT**S**^{8}ARRSYAS
			RITSARR**S**^{12}YASETVV
			LGTIPRL**S**^{36}LSRMTPP
Heat shock protein 8	4.8	3.4	GIDLGTT**Y**^{15}SCVGVFQ
			GVPQIEV**T**^{477}FDIDANG
Transcriptional activator protein pur-alpha (TAPP)	2.5	1.0	SRLTLSM**S**^{104†}VAVEFRD
			QEETTAA**T**^{309†}LLLQGEE

*All possible phosphosites are cited from the PhosphoSite™ website unless otherwise indicated (http://www.phosphosite.org). †These two possible phosphosites are predicted by Scansite motif scan (http://scansite.mit.edu/motifscan_id.phtml).

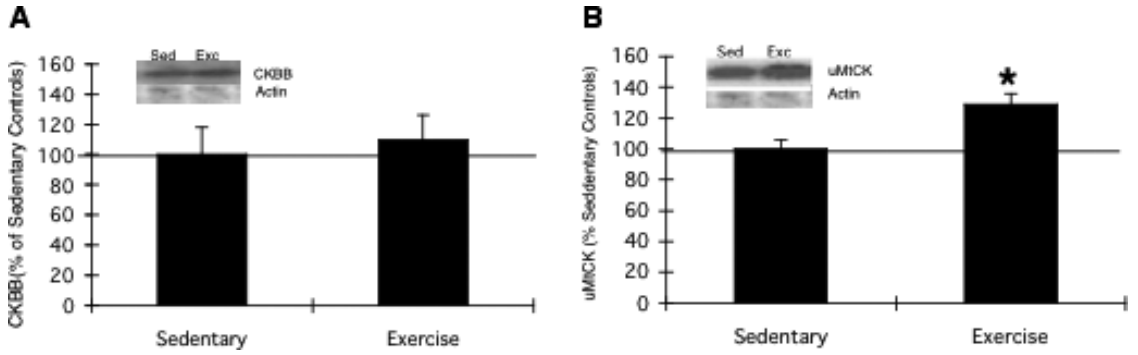

FIG. 2. Western blot of brain-type (CKBB) (A) and ubiquitous mitochondrial (uMtCK) (B) creatine kinases. Consistent with two-dimensional gel electrophoresis analysis, exercise significantly increased uMtCK expression without significantly affecting the cytosolic isoform CKBB. Representative immunoblots for CKBB and uMtCK, with actin as the control, are shown for the exercise (Exc) and sedentary (Sed) control groups. Results are displayed as percentages of sedentary levels (represented by the 100% line). Data are expressed as the mean ± SEM ($n = 5$ per group, *t*-test, $^*P < 0.05$).

Mitochondrial ATP synthases

The mitochondrial ATP synthase complex is composed of F1 and F0 units. The F1 mitochondrial ATPase forms the catalytic component whereas the F0 unit functions as the membrane proton channel. Exercise significantly affected the expression of the mitochondrial ATP synthase by increasing both the alpha and beta chains of the F1 unit. We found three protein spots for the alpha form, with increased fold changes from 1.9 to 3.0 (Table 2). Two protein spots for the beta form expressed increased fold changes of 1.8 and 1.9 (Table 2).

Enzymes of energy transduction

Creatine kinases reversibly catalyse the transfer of phosphate between ATP and creatine as well as various other phosphogens. We found that the increase of uMtCK was pronounced following exercise, showing a fold change of 1.7 (Table 2). However, the brain cytosolic isoform, CKBB, was not significantly changed with exercise, with a protein fold change of 1.5. Western blot analysis confirmed that exercise significantly increased the levels of uMtCK (129 ± 7.2%) above sedentary control levels (100 ± 6.2%) without significantly altering the levels of CKBB (110 ± 16.5%) from those of sedentary control animals (100 ± 18.2%; Fig. 2).

Proteins involved in synaptic plasticity

Cytoskeletal proteins

Cytoskeletal proteins form the structural integrity necessary for synaptic plasticity. Our results show that exercise up-regulated cytoskeletal proteins in the hippocampus. We found a protein fold increase in β-tubulin of 1.9 and α-internexin of 1.7 (Table 3). Moreover, the phosphorylated form of β-tubulin was differentially expressed after exercise with a fold change of 2.0 (Table 4). We found two spots for glial fibrillary acidic protein (GFAP), both exhibiting a fold increase of 1.7 (Table 3). Additionally, Pro-Q Diamond fluorescent staining showed that the phosphorylation of GFAP was also significantly enhanced with exercise, with an intensity fold change of 1.7 (Table 4, Fig. 3). Interestingly, Pro-Q Diamond fluorescent staining showed that the phosphorylated form of NFL was increased with exercise, exhibiting a fold change of 2.7, even though its total protein levels were not differentially increased by exercise (protein fold 1.1; Table 4). We also found an increased fold change in the phosphorylated form of the TAPP following exercise, exhibiting a protein fold increase of 2.5 (Table 4; Fig. 3).

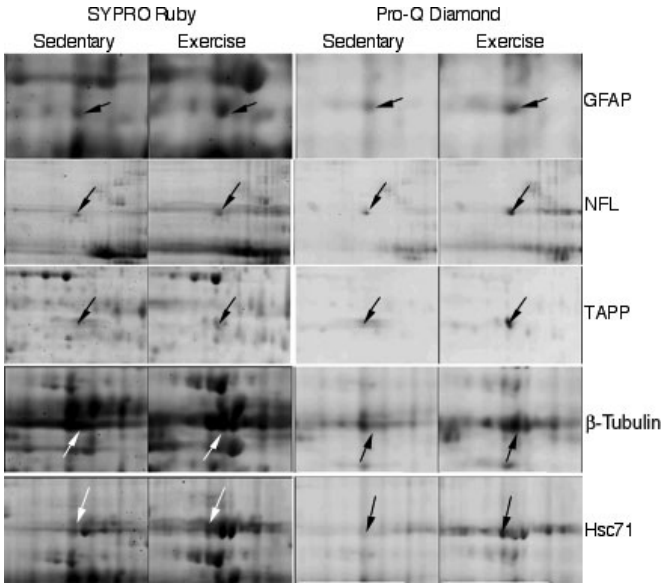

FIG. 3. Differential change in the pattern of total (SYPRO Ruby-stained) and phosphorylated (Pro-Q Diamond-stained) hippocampal proteins induced by voluntary exercise. GFAP, glial fibrillary acidic protein; NFL, neurofilament light polypeptide; TAPP, transcriptional activator protein pur-alpha; Hsc71, heat shock protein 8.

Chaperones

Chaperones are a category of molecules that assist in protein folding and oligomeric assembly in which many heat shock proteins have been categorized as neural plasticity-related molecules (Nedivi *et al.*, 1993; Hong *et al.*, 2004). In this experiment, we found that voluntary exercise increased the expression of heat shock protein 8, heat shock 60-kDa protein 1 and chaperonin-containing t-complex peptide 1 (TCP-1) (CCT) (beta subunit) in the hippocampus (Table 3). Neuronal protein 22 (NP22), a newly identified chaperone-like protein, was also found to be significantly up-regulated by exercise with a fold change of 2.2 (Table 3). Heat shock 60-kDa protein 1 exhibited a fold change of 2.0. CCT (beta subunit) displayed a fold change of 1.9 (Table 3). Of the chaperones that we found to be increased with exercise, hippocampal heat shock protein 8 exhibited the largest fold change of 3.4 (Table 3) and was significantly phosphorylated following exercise, exhibiting a fold change of 4.8 (Table 4).

Discussion

Studies have shown that exercise is capable of inducing molecular mechanisms that support cognitive function. However, it is not well understood how exercise orchestrates the action of multiple factors at the protein level, in particular those related to energy metabolism and synaptic plasticity. Proteomics provided us with a powerful tool to profile protein expression and modification on a global level. After analysing many protein spots of relatively high abundance on two-dimensional gel electrophoresis gels from the hippocampus, it was particularly evident that the proteins differentially expressed by exercise were associated with energy metabolism and neuronal plasticity.

Exercise up-regulates proteins involved in energy metabolism

Proteins involved in glucose catabolism

Short-term exercise up-regulates enzymes in the energy-consuming and energy-generating steps of glycolysis, fructose-bisphosphate aldolase C and PGK-1, respectively. Aldolase C catalyses fructose 1,6-bisphosphate to glycerone phosphate and glyceraldehyde 3-phosphate (Penhoet & Rutter, 1975). PGK-1 is involved in the second step of the second phase of glycolysis, to convert 1,3-diphosphoglycerate to 3-phosphoglycerate while forming one molecule of ATP. ATP from PGK-1 can be used to ensure maximum glutamate accumulation into synaptic vesicles (Fig. 4). A reduction in PGK-1 is associated with age-related impaired cellular processes (Oliver *et al.*, 1987). It has been found that caloric restriction, which has been shown to have a beneficial effect on synaptic plasticity and cognitive function, increases PGK-1 levels in the hippocampus (Poon *et al.*, 2006).

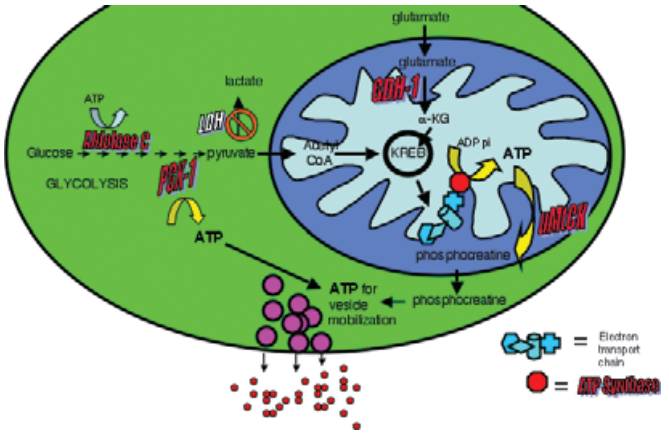

FIG. 4. Potential mechanism by which exercise modulates proteins involved in energy metabolism and synaptic plasticity in the hippocampus. Exercise up-regulates proteins involved in four stages of energy metabolism: glucose catabolism, ATP synthesis, energy transduction and glutamate turnover. The increase in the enzymes involved in the two stages of glycolysis, fructose-bisphosphate aldolase C and phosphoglycerate kinase 1 (PGK-1), provides more substrate for the Kreb cycle. Likewise, the breakdown of glutamate to α-ketoglutarate by glutamate dehydrogenase (GDH)1 not only increases the turnover of glutamate but also provides another entry point to the Kreb cycle. An increase in the alpha and beta forms of ATP synthase, which form the complex for ATP synthesis, may constitute a mechanism by which exercise increases energy production. Any increased mitochondrial ATP supply can be transferred to areas in need of energy in the cytoplasm by phosphocreatine produced through ubiquitous mitochondrial creatine kinase (uMtCK). Increased ATP generated from the mitochondrion and from glycolysis may be used to advance vesicle mobilization necessary for high synaptic transmission. Excess glutamate can then be recycled by GDH-1 into the Kreb cycle to perpetuate ATP production. Proteins increased by exercise are shown in red. Lactate dehydrogenase (LDH) is shown in white to indicate the possible decline in the reliance on the pyruvate to lactate conversion for energy production during exercise. α-KG, α-ketoglutarate.

Proteins involved in ATP synthesis

Proteomics revealed that exercise increased ATP synthase alpha and beta forms, members of the F1 synthase enzymatic complex that synthesizes ATP in oxidative phosphorylation (Poon *et al.*, 2006). ATP synthase may contribute to the metabolic malfunction (consisting of mitochondrial dysfunction and glutamate dysregulation) characterizing age-related cognitive impairment (Poon *et al.*, 2006).

Our results showed that exercise decreased lactate dehydrogenase, which exhibited a protein fold decrease of 1.8 (Table 2). Lactate dehydrogenase provides another pathway to energy production, an alternative to the Kreb cycle, by catalysing the interconversion of pyruvate to lactate with the concomitant interconversion of NADH and NAD+. Our finding that exercise produced a decrease in lactate dehydrogenase may indicate that the lactate is not a primary source of energy production (Fig. 4). Given that lactate dehydrogenase levels have been used as a measure of neuronal plasma membrane damage for the effects of oxidative stress (Laev *et al.*, 1993; Cimarosti *et al.*, 2005), our findings may also indicate that exercise has a beneficial affect on neuronal integrity.

Proteins involved in energy transduction

Exercise up-regulated uMtCK, a finding concordant with evidence that uMtCK is modulated by neuronal activity (Boero *et al.*, 2003). uMtCK is functionally coupled to oxidative phosphorylation, as mitochondrial-derived ATP is preferentially used by uMtCK to transfer high-energy phosphates to creatine (Jacobus & Lehninger, 1973; Rojo *et al.*, 1991). The resultant phosphocreatine acts as the cytosolic transport and storage form of energy; it is used to regenerate ATP from cytosolic ADP (Rango *et al.*, 1997), which is essential for maintaining the ATP supply during high synaptic transmission (Whittingham & Lipton, 1981; Fig. 4). Increased energy demands modify uMtCK distribution and expression, thus providing a mechanism of energy transduction in which ATP synthesized in the mitochondrion is transferred to sites of ATP utilization.

Proteins involved in glutamate turnover

Exercise increased the inner mitochondrial enzyme GDH-1. GDH-1 eliminates excitotoxic glutamate in the brain by oxidizing glutamate to α-ketoglutarate (Cooper & Meister, 1985). GDH activity is prominent in synaptic terminals (Lai *et al.*, 1977, 1986; Lai & Clark, 1979; Erecinska & Nelson, 1990; McKenna *et al.*, 1993), where much of the endogenous glutamate enters into the Kreb cycle primarily through GDH (McKenna *et al.*, 2000; Fig. 4). Widely expressed throughout the hippocampus, GDH may support learning and memory by increasing glutamate turnover (Sakimura *et al.*, 1995; McHugh *et al.*, 1996; Cavallaro *et al.*, 1997; Fig. 4). In fact, GDH mRNA levels increase in the hippocampus after spatial learning (Cavallaro *et al.*, 1997).

Exercise up-regulates proteins involved in synaptic plasticity

Expanding the cellular network: cytoskeletal proteins

Studies have shown that exercise induces neurogenesis in the adult brain (van Praag *et al.*, 1999, 2005). In support of this, our results indicate that exercise increases a diverse set of proteins related to cytoskeletal function and neuronal development, i.e. α-internexin,

phosphorylated NFL, phosphorylated TAPP, β-tubulin, GFAP (total and phosphorylated) and NP22 (Fig. 3, Table 4).

α-internexin, another neurone-specific protein (Evans *et al.*, 2002), is critical for neuronal development (Kaplan *et al.*, 1990; Fliegner *et al.*, 1994) as it stabilizes neurones and their processes by providing a scaffold for the assembly of other intermediate filament proteins (Fliegner *et al.*, 1994). It is notable that we found increases in both α-internexin and phosphorylated NFL after exercise, as both may indicate the presence of new neurones. In the developing brain, α-internexin and NFL are sequentially expressed, with the presence of NFL and other intermediate filaments designating different states of neuronal maturation (Shaw & Weber, 1982; Carden *et al.*, 1987; Kaplan *et al.*, 1990). α-internexin may also be important for energy homeostasis, as it is significantly correlated with glutamate transporter levels in the energetically compromised postinjury brain (Yi *et al.*, 2005). The contribution of α-internexin to cytoskeletal integrity is important for axonal calibre and nerve conduction speed, intimating that a dysfunction in this and other intermediate filaments may contribute to the memory deficits seen in diseases characterized by cognitive impairment (Lalonde & Strazielle, 2003).

Exercise increased the phosphorylated form of the TAPP. TAPP regulates the transcription of several genes including myelin basic protein (Tretiakova *et al.*, 1999) and the nicotinic acetylcholine receptor β4 gene (Du *et al.*, 1997; Melnikova *et al.*, 2000). Knockout of pur-alpha in mice has been found to produce neural-specific abnormalities associated with abnormal cellular proliferation (Khalili *et al.*, 2003).

Increases in GFAP may indicate the action of exercise on astrocyte proliferation or neurogenesis. GFAP is generally used as a hallmark of mature astrocytes (Raju *et al.*, 1981), although in the hippocampal subventricular zone, GFAP-positive cells can generate both neurones and glia (Doetsch *et al.*, 1999). GFAP is associated with cognitive function and synaptic plasticity, as both inhibitory avoidance and habituation alter the *in vitro* phosphorylation of NFL and GFAP (Schroder *et al.*, 1997).

Neuronal protein 22 is a novel neurone-specific protein that has putative cytoarchitectural functions as it holds an actin-binding domain and shares sequence homology with proteins, calponin and smooth muscle 22α (SM22α), that bind the cytoskeleton (Ito *et al.*, 2005). NP22 colocalizes with all three major components of neuronal microtubules, tau, α-tubulin and MAP2 (Depaz *et al.*, 2005), and with F-actin (Depaz *et al.*, 2005), which is highly expressed in dendrites, synapses, dendritic spines and postsynaptic densities (Markham & Fifkova, 1986; Fifkova & Morales, 1992; Adam & Matus, 1996; Halpain, 2000). These qualifications, coupled with the finding that NP22 has a protein kinase C binding domain for extracellular-induced activation (Niki *et al.*, 1996), suggest that NP22 may be involved in mediating changes in synaptic plasticity.

Molecular chaperones

Molecular chaperones are a category of proteins that are involved in protein import, folding and assembly (Beckmann *et al.*, 1990). Their functions extend to combating oxidative stress and apoptosis and facilitating synaptic activity, as illustrated by the chaperones that we found to be up-regulated by exercise, i.e. CCT, heat shock 60-kDa protein 1 and heat shock protein 8.

CCT belongs to a subclass of the chaperonin family that facilitates the folding of large non-native proteins (Kubota *et al.*, 1995; Hartl & Hayer-Hartl, 2002). CCT maintains cytoarchitecture by correcting the three-dimensional structure of cytoskeletal proteins such as actin, tubulin and other polypeptides in the cytosol (Sternlicht *et al.*, 1993). CCT may also help the cell withstand oxidative stress by promoting cellular homeostasis (Kane *et al.*, 2004). In fact, the neurodegenerative diseases Alzheimer's and Parkinson's, with an aetiology of oxidative stress (Mariani *et al.*, 2005), have been linked to CCT down-regulation (Lopez Salon *et al.*, 2000; McNaught & Jenner, 2001).

Heat shock 60-kDa protein 1 is a mitochondrial chaperone that may protect mitochondria from oxidative stress by facilitating the proper assembly of mitochondrial proteins (Bozner *et al.*, 2002; Boyd-Kimball *et al.*, 2005). Heat shock 60-kDa protein 1 has anti-apoptotic functions (Lin *et al.*, 2001) and its expression is decreased in Alzheimer's disease (Yoo *et al.*, 2001).

Heat shock protein 8, otherwise known as heat shock cognate 71-kDa protein, belongs to the heat shock protein 70 family and has anti-apoptotic functions. It is strongly expressed in neurones under homeostatic conditions but is up-regulated in astrocytes during brain injury (Kanninen *et al.*, 2004). Heat shock protein 8 has been reported to be oxidatively modified in Alzheimer's disease (Castegna *et al.*, 2002).

Conclusions

By using a proteomics approach, this study has demonstrated that exercise affects a diverse network of proteins related to energy metabolism and synaptic plasticity in the hippocampus, a brain region central to cognitive function. Exercise increased four different aspects of energy metabolism (Fig. 4). An up-regulation of glycolytic enzymes may indicate increased glucose catabolism. An increase in GDH-1 increases glutamate turnover and provides another entry point into the Kreb cycle. Oxidative phosphorylation also seems to be affected by exercise as the ATP synthase machinery was increased. Finally, by up-regulating uMtCK, exercise may increase the cellular bio-availability of mitochondrial-produced ATP.

An obvious question arising from these findings, to be explored by future research, is how exercise enables energy metabolism to interact with synaptic plasticity to affect brain functions, i.e. spatial learning and memory in the hippocampus. Glucose catabolism may support brain function as glucose utilization is directly correlated with cognitive function (McNay *et al.*, 2000). Furthermore, the increased production and transduction of mitochondrial ATP may be used for vesicle mobilization, especially under high synaptic transmission (Verstreken *et al.*, 2005; Fig. 4). Possibly, the binding of cytoskeletal proteins to enzymes of energy metabolism may modulate cellular functions (Durrieu *et al.*, 1987; Shearwin *et al.*, 1990; Knull & Walsh, 1992). Finally, as mitochondrial function may significantly increase during exercise, chaperones such as heat shock 60-kDa protein 1 and CCT may be essential for protecting the mitochondrion and cell from oxidative stress. Notwithstanding this, it is notable that most of the proteins up-regulated by exercise, whether metabolic, cytoskeletal or chaperone related, have been implicated in supporting cognitive function.

Acknowledgements

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Abbreviations

CCT, chaperonin-containing TCP-1; CKBB, brain-type creatine kinase; GDH, glutamate dehydrogenase; GFAP, glial fibrillary acidic protein; NFL, neurofilament light polypeptide; NP22, neuronal protein 22; PGK-1, phosphoglycerate kinase 1; TAPP, transcriptional activator protein pur-alpha; TCP-1, t-complex peptide 1; uMtCK, ubiquitous mitochondrial creatine kinase.

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