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Differential effects of acute and chronic exercise on plasticity-related genes in the rat hippocampus revealed by microarray

Raffaella Molteni,[2] Zhe Ying[2] and Fernando Gómez-Pinilla[1,2]
[1]Division of Neurosurgery, UCLA Brain Injury Research Center and
[2]Department of Physiological Science, UCLA, 621 Charles E. Young Drive Los Angeles 90095, California, USA

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Abstract

Studies were performed to determine the effects of acute and chronic voluntary periods of exercise on the expression of hippocampal genes. RNAs from rodents exposed to a running wheel for 3, 7 and 28 days were examined using a microarray with 1176 cDNAs expressed primarily in the brain. The expression of selected genes was quantified by Taqman RT-PCR or RNase protection assay. The largest up-regulation was observed in genes involved with synaptic trafficking (synapsin I, synaptotagmin and syntaxin); signal transduction pathways (Ca^{2+}/calmodulin-dependent protein kinase II, CaM-KII; mitogen-activated/extracellular signal-regulated protein kinase, MAP-K/ERK I and II; protein kinase C, PKC-δ) or transcription regulators (cyclic AMP response element binding protein, CREB). Genes associated with the glutamatergic system were up-regulated (*N*-methyl-D-aspartate receptor, NMDAR-2A and NMDAR-2B and excitatory amino acid carrier 1, EAAC1), while genes related to the gamma-aminobutyric acid (GABA) system were down-regulated ($GABA_A$ receptor, glutamate decarboxylase GAD65). Brain-derived neurotrophic factor (BDNF) was the only trophic factor whose gene was consistently up-regulated at all timepoints. These results, together with the fact that most of the genes up-regulated have a recognized interaction with BDNF, suggest a central role for BDNF on the effects of exercise on brain plasticity. The temporal profile of gene expression seems to delineate a mechanism by which specific molecular pathways are activated after exercise performance. For example, the CaM-K signal system seems to be active during acute and chronic periods of exercise, while the MAP-K/ERK system seems more important during long-term exercise.

Introduction

Evidence accumulated over many decades illustrates the beneficial action of physical activity in maintaining and improving neural function in humans (Kramer *et al*., 1999) and animals (Fordyce & Farrar, 1991). Exercise has been shown to reduce the cognitive decline during ageing (Laurin *et al*., 2001), to help recover functional loss after CNS damage (Mattson, 2000) andto promote neurogenesis in the adult hippocampus (van Praag *et al*., 1999). The limited understanding of the mechanisms through which exercise impacts brain plasticity and function has hampered major advances on the implementation of exercise as a therapeutical tool. Recent studies showing the capacity of exercise to induce specific trophic factors in select brain regions (Neeper *et al*., 1995; Gomez-Pinilla *et al*., 1997) have offered leads to initiate a systematic evaluation of the action of exercise on neural function.

Exercise induces BDNF mRNA (Neeper *et al*., 1995) and protein (Vaynman *et al*., 2001) in the hippocampus. In addition to protecting neurons from various types of insults (Larsson *et al*., 1999), BDNF promotes adult neurogenesis (Benraiss *et al*., 2001; Pencea *et al*., 2001) and increases synaptic efficacy (Poo, 2001). It is evident that BDNF is a critical component of the molecular mechanisms by which exercise impacts neuroplasticity. However, given the broad physiological meaning of exercise, it is likely that multiple molecular systems can be involved.

We have pursued studies to identify molecular systems and possible pathways involved with the action of exercise on the brain, and to determine the place that BDNF occupies in these events. We have used cDNA array technology to simultaneously screen over 1000 genes, that are expressed mostly in the brain, encoding transcription factors, intracellular signal transduction modulators, neurotransmitters receptors and growth factors, apoptotic proteins, metabolic protein, protein turnover andcell–cell communication molecules.

A standing question for planning the design of studies using the therapeutical potential of exercise is whether exercise provided for a short period can have the same benefit as long-term exercise. Therefore, in order to have a global dimension of the action of exercise on neural function, it is crucial to determine the capacity that exercise has to orchestrate multiple molecular systems over time. Accordingly, we have evaluated the effects of exercise provided for the period of a few days or weeks on the expression of hippocampal genes. We focused this study on the hippocampus because of the potential of exercise to modify molecular events governing learning and memory, and because the hippocampus is the locus of critical aspects of neuronal plasticity such as adult neurogenesis (Gross, 2000).

Correspondence: Dr Fernando Gómez-Pinilla, [2]Department of Physiological Science, as above.
E-mail: Fgomezpi@ucla.edu

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Materials and methods

Animals and exercise

Male Sprague–Dawley rats, 3 months of age (Charles River, CA, USA) were housed singly in standard polyethylene cages with food and water *ad libitum*, and with a 12-h light : 12-h dark cycle. After 1 week of acclimation, the animals were assigned randomly to a control ($n = 5$) or exercise ($n = 5$, each time point) group. Exercising animals were placed in standard cages equipped with running wheels (diameter, 31.8 cm, width, 10 cm; Nalgene Nunc International, NY, USA) that rotated against a resistance of 100 g. Animals included in the study had to run at least 5 km per night. Sedentary animals were left undisturbed in their homecages. Wheel revolutions were recorded by computer using VitalViewer software (Mini Mitter Company, Inc., OR, USA). Rats were killed by decapitation at 07.00 h after 3, 7 and 28 days of exercise. The hippocampus was dissected out rapidly, frozen on dry ice and stored at –70 °C until processed. All experiments were performed in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of California at Los Angeles, Animals Research Committee.

CDNA microarray

Total RNA was isolated by using RNA STAT-60 kit (TEL-TEST, Inc., Friendswood, TX) according to manufacturer's instructions and quantified by spectrophotometric analysis. The same amount of hippocampal RNA from each individual animal (4 μg) within the same experimental group was pooled. Pooled total RNA (3 μg) for each experimental group was converted into ^{32}P-cDNA, and used to hybridize rat cDNA microarrays (Atlas rat array, Clontech, CA, USA) according to the manufacturer's instructions. Briefly, each membrane was prehybridized for 30 min at 68 °C in 5 mL of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization with radiolabelled cDNA probes ($\approx 1 \times 10^6$ c.p.m./mL) was carried out overnight in 5 mL of ExpressHyb at 68 °C. Membranes were washed with continuous agitation at 68 °C in 2× SSC, 1% SDS and then in 0.1× SSC, 0.5% SDS, plastic-wrapped, exposed to a phosphorimager screen (Molecular Dynamics, Amersham Biosciences, CA, USA) for 3–5 h and scanned with PhosphorImager SI 445 (Molecular Dynamics).

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

The mRNAs for BDNF, NT-3, Synapsin I, TrkB and CREB were measured in the total RNA obtained from individual animal by TaqMan real-time quantitative RT-PCR using an ABI PRISM 7700 Sequence detection system (Perkin-Elmer, Applied Biosystems, CA, USA) and TaqMan EZ RT-PCR Core reagents (Perkin-Elmer, NJ, USA). This system detects directly the increase in fluorescence of a dye-labelled DNA probe specific for each factor under study plus a probe specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene used as an endogenous control. The sequences of probes, forward and reverse primers (Integrated DNA Technologies, IA, USA) were as follows.

BDNF
(5′-AGTCATTTGCGCACAACTTTAAAAGTCTGCATT-3′;
forward, 5′-GGACATATCCATGACCAGAAAGAAA-3′;
reverse, 5′-GCAACAAACCACAACATTATCGAG-3′);
NT-3
(5′-TGACCGACAAGTCCTCAGCCATTGAC-3′;
forward, 5′-TGTGACAGTGAGAGCCTGTGG-3′;
reverse, 5′-TGTAACCTGGTGTCCCCGAA-3′);
TrkB
(5′-TGCACGTCTGGCCGCTCCTAACC-3′;
forward, 5′-CCCAATTGTGGTCTGCCG-3′;
reverse, 5′-CTTCCCTTCCTCCACCGTG-3′);
synapsin I
(5′-CATGGCACGTAATGGAGACTACCGCA-3′;
forward, 5′-CCGCCAGCTGCCTTC-3′;
reverse, 5′-TGCAGCCCAATGACCAAA-3′);
CREB
(5′-CATGGCACGTAATGGAGACTACCGCA-3′;
forward, 5′-CCGCCAGCATGCCTTC-3′;
reverse, 5′-TGCAGCCCAATGACCAAA-3′).

The RT-reaction conditions were 2 min at 50 °C as initial step to activate uracil glycosylase (UNG), followed by 30 min at 60 °C as reverse transcription and completed by UNG-deactivation at 95 °C for 5 min. The 40 cycles of two-step PCR-reaction conditions were 20 s at 94 °C and 1 min at 62 °C.

RNase protection assay

The cDNAs for the NMDA-R subunits and for the glutamate transporters were arranged as described previously (Riva *et al.*, 1994). The cRNA probes and the relative protected fragment (p.f.) were the following: NR-1 = 438, p.f. = 414; NR-2A = 187, p.f. = 171; NR-2B = 310, p.f. = 264; EAAC1 = 534, p.f. = 500; GLAST = 375, p.f. = 355; GLT1 = 438, p.f. = 414; GAPDH = 376, p.f. = 316. The RNase protection assay was performed on a 10-μg sample of total RNA (Riva *et al.*, 1997). Briefly, total RNA obtained from each individual animal was dissolved in 20 μL of hybridization solution containing ^{32}P-labelled cRNA probes (50 000 c.p.m. of GAPDH used as internal standard and 150 000 c.p.m. of each probe under study; specific activity $> 10^8$ c.p.m./μL). After being heated at 85 °C for 10 min, the cRNA probes were allowed to hybridize the endogenous RNAs at 45 °C overnight. The protected fragments were visualized by autoradiography. The levels of mRNA were calculated by measuring the optical density using Quantity One software (Bio-Rad Laboratories, CA, USA).

Analyses of gene array data

The 16-bit phosphoimager file for each membrane was converted to a TIF file and analysed with AtlasImage 1.5 software program (Clontech). The signal in each spot of the membrane was determined from the pixel density and corrected for background. Signal was normalized using five housekeeping genes within the array (polyubiquitin, glyceraldheyde 3-phosphate deydrogenase, α-tubulin, β-actin, ribosomal protein S29). For each comparison (e.g. control relative to exercise) and for each cDNA represented in the array, a ratio (exercise : sedentary group) was calculated. Only genes that showed an expression ratio (exercise group : sedentary group) of $\geqslant 1.8$ or $\leqslant 0.5$ were considered to be significantly different in the two groups, i.e. to be differentially expressed.

Statistical analyses

For real-time RT-PCR and for RNase protection assay, GAPDH was employed as an internal standard, as its expression was not altered by exercise. For quantification of TaqMan RT-PCR results, fluorescent signal intensities were plotted against the number of PCR cycles on a semilogaritmic scale. The amplification cycle at which the first significant increase of fluorescence occurred was designed as the

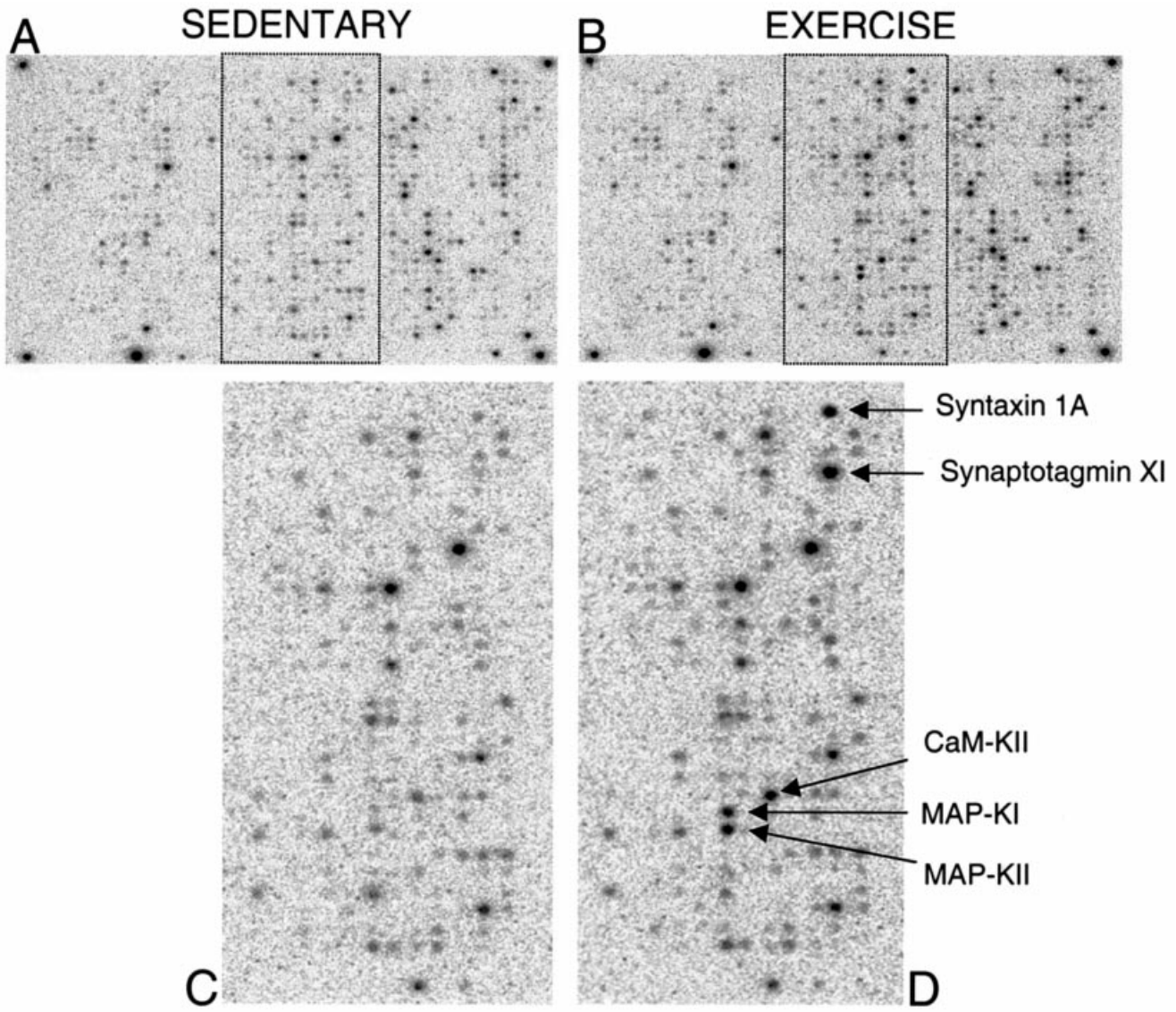

FIG. 1. Representative posthybridization array membranes showing differential expression of genes in the hippocampus of rats engaged in (B) voluntary wheel running for 28 days vs. (A) sedentary controls. P32-labelled cDNA were generated from the total RNA samples, and used to hybridize the arrays (See Materials and method). Bottom figures are magnifications from the inserts shown above for (C) sedentary and (D) exercise, respectively. Membranes were exposed to a phosphorimager screen for 4 h and analysed by scanning with a PhosphorImager to derive results shown in Tables 1–3. Sample arrows highlight cDNAs for which mRNA abundances significantly differ between sedentary and exercise groups.

threshold cycle (C_T). The C_T value of each sample was then compared to those of the internal standard. This process was fully automated and carried out with ABI sequence detector software version 1.6.3 (Perkin Elmer). Taqman RT-PCR values for GAPDH were subtracted from BDNF, NT-3, synapsin I, TrkB or CREB values for each animal. The resulting corrected values were used to make comparisons across the different experimental groups. For quantification of RNase protection assay results, the mean corrected values for the mRNA levels were computed for the control (sedentary) and experimental (exercise) group for each animal at each time point. The results of Taqman RT-PCR and RNase protection assay were analysed using one-way analysis of variance (ANOVA), followed by the Sheffe *t*-test for multiple comparisons. Statistical differences were considered significant at $P < 0.05$. The results were expressed as the mean percentage of control values (for graphic clarity) and represent the mean ± standard error of the mean (SEM) of five independent determinations.

Results

The rat cDNA microarray used for the present study contains genes encoding transcription factors, intracellular signal transduction modulators, neurotransmitter and growth factor receptors, molecules involved with apoptosis, metabolism, protein turnover and cell–cell communication. Representative microarray hybridization results are shown for sedentary and 28 days of exercise in Fig. 1. There were several genes whose expression ratio were 0.5–0.8 or 1.3–1.8, at any of the three time-points. These 'alterations' were considered only as a 'tendency to change', i.e. marginal, and they are shown in Table 1 for up-regulation and Table 2 for downregulation. Genes showing an expression ratio (exercise group : sedentary group) of $\geqslant 1.8$ or $\leqslant 0.5$ for at least one time point, were considered to be differentially expressed, i.e. to show a significant difference between the two groups, and are shown in Table 3. For illustrative purposes, these genes have been grouped in Table 3 according to their biochemical

TABLE 1. Hippocampal genes with marginal up-regulation after exercise; the expression ratio (exercise : sedentary group) is presented

Gene alternative names (and common abbreviations)	Expression ratio (exercise : sedentary)		
	3 days	7 days	28 days
14-3-3 protein epsilon; PKC inhibitor protein-1; KCIP-1; mitochondrial import stimulation factor L subunit	1.16	1.49	1.21
40S ribosomal protein S11	1.38	1.32	1.27
5-hydroxytryptamine (serotonin) receptor 5B; 5HT$_{5b}$	1.47	1.59	1.34
60S ribosomal protein L19 (RPL19)	1.31	1.27	1.27
60S ribosomal protein L21	1.66	1.67	1.11
A-raf proto-oncogene	1.23	1.22	1.56
Adenylyl cyclase type II	1.31	1.46	1.45
ADP-ribosylation factor 3 (ARF3)	1.34	1.20	1.27
ADP-ribosylation factor 5 (ARF5)	1.36	1.12	1.13
Advanced glycosylation end product-specific receptor precursor (AGER); receptor for advanced glycosylation end products (RAGE)	1.64	1.77	1.24
ATPase, calcium, brain	1.41	1.44	1.01
c-H-ras proto-oncogene; transforming G-protein p21	1.41	1.43	1.48
c-jun proto-oncogene; transcription factor AP-1; RJG-9	1.26	1.34	1.56
c-K-ras 2b proto-oncogene; transforming G-protein p21	1.65	1.40	1.48
C-type natriuretic peptide precursor (CNP; NPPC)	1.05	1.15	1.30
Calcium binding protein 2 (CABP2); endoplasmic reticulum stress protein (ERP72); protein disulphide isomerase-related protein precursor	1.20	1.45	1.20
Calmodulin (CALM; CAM)	1.24	1.57	1.23
cAMP-dependent protein kinase type I-alpha regulatory chain	1.32	1.31	1.67
Carboxypeptidase E; carboxipeptidase H	1.69	1.33	1.00
Clusterin (CLU); testosterone-repressed prostate message 2 (TRPM2); apolipoprotein J; sulphated glycoprotein 2 (SGP2); dimeric acid glycoprotein (DAG)	1.56	1.25	1.04
Cofilin	1.36	1.12	1.56
Copper-zinc-containing superoxide dismutase 1 (Cu-Zn SOD1)	1.20	1.39	1.20
Cytochrome c oxidase subunit Vb & VIa precursor (COX5B)	1.32	1.45	1.23
Cytochrome c oxidase, subunit IV, mitochondrial	1.20	1.34	1.54
Cytochrome c oxidase, subunit VIIIh	1.68	1.50	1.08
Elongation factor 2 (EF2)	1.67	1.71	1.62
Fatty acid binding protein, brain	1.24	1.62	1.56
Fibroblast ADP/ATP carrier protein; ADP/ATP translocase 2; adenine nucleotide translocator 2 (ANT2)	1.36	1.23	1.13
Fte-1; putative v-fos transformation effector protein; yeast mitochondrial protein import homologue; 40S ribosomal protein S3A; RPS3A	1.04	1.22	1.34
G1/S-specific cyclin D2 (CCND2); vin-1 proto-oncogene	1.31	1.00	1.00
Glutamate receptor 1 precursor (GluR-1); GluR-A; GluR-K1	1.37	1.42	1.03
Glutamate/aspartate transporter, cochleae	1.35	1.23	1.12
Guanine nucleotide-binding protein alpha 12 subunit (G alpha 12, GNA12)	1.30	1.23	1.22
Guanine nucleotide-binding protein G(O) alpha subunit (GNAO)	1.04	1.12	1.77
Guanine nucleotide-binding regulatory, alpha subunit	1.16	1.23	1.44
Hypoxanthine-guanine phosphoribosyltransferase (HPRT)	1.32	1.21	1.22
Id-3; DNA-binding protein inhibitor; HLH protein	1.27	1.07	1.00
Inositol 1,4,5-triphosphate 3-kinase receptor 2 (INSP3R)	1.36	1.54	1.22
Microglobulin; beta-2-microglobulin + prostaglandin receptor F2a	1.15	1.45	1.68
Mitochondrial ATP synthase beta subunit precursor (ATP5B)	1.00	1.40	1.37
Muscle 6-phosphofructokinase (PFKM); phosphofructokinase 1; phosphohexokinase; phosphofructo-1-kinase A	1.53	1.60	1.06
Myelin basic protein s (mbp s)	1.18	1.52	1.63
Myelin proteolipid protein (PLP); DM-20; lipophilin	1.35	1.45	1.77
Na+/K + ATPase alpha 1 subunit	1.36	1.55	1.75
Neural adhesion molecule F3, rat neural adhesion molecule F3, complete CDs.	1.43	1.47	1.68
Neurexin I-beta precursor, non-processed neurexin I-beta and alpha Synaptic cell surface proteins	1.49	1.63	1.09
Neuroendrocrine protein 7B2 precursor; secretogranin V; SGNE1	1.30	1.56	1.04
Neuromodulin; axonal membrane protein GAP43; PP46; B-50; protein F1; calmodulin-binding protein P-57	1.35	1.35	1.35
Neuron-specific enolase (NSE); gamma enolase (EC 4.2.1.11); 2-phospho-D-glycerate hydrolyase	1.16	1.42	1.31
Neuronal acetylcholine receptor protein beta 2 subunit precursor (nonalpha 1, CHRNB2, ACRB2)	1.37	1.23	1.34
Neuronal pentraxin receptor	1.30	1.29	1.04
Neurotrophin 3 (NT3)	1.20	1.38	1.36
Nm23-M2; nucleoside diphosphate kinase B; metastasis-reducing protein; c-myc-related transcription factor, nucleoside diphosphate kinase A (EC 2.7.4.6)	1.43	1.74	1.64
N-methyl-D-aspartate-receptor subtype 1 (NMDAR1)	1.68	1.23	1.05
Non-processed neurexin II-beta major, neurexin II-beta-a precursor Non-processed neurexin II-alpha, neurexin II-alpha-B precursor	1.30	1.60	1.30
Nuclear tyrosine phosphatase; PRL-1; affects cell growth	1.66	1.58	1.32
NVP-2; neural visinin-like Ca^{2+}-binding protein, visinin-like protein 2 (vilip-2); neural visinin-like protein 2 (NVL-2, NVP-2).	1.65	1.42	1.15

TABLE 1 (continued)

Gene alternative names (and common abbreviations)	Expression ratio (exercise : sedentary)		
	3 days	7 days	28 days
NVP; neural visinin-like Ca^{2+}-binding protein, visinin-like protein 1 (vilip-1); neural visinin-like protein 1 (NVL-1, NVP-1); 21 KD CABP	1.17	1.43	1.34
PDGF-associated protein	1.32	1.24	1.31
Potassium channel RCK4, subunit, putative	1.28	1.37	1.44
Potassium channel-like protein KATP2, beta cell	1.39	1.36	1.55
Protein kinase C beta-I type (PKC-beta I) + protein kinase C beta-II type (PKC-beta II)	1.10	1.10	1.59
Protein kinase C zeta type (PKC-zeta)	1.30	1.19	1.02
rab12, ras-related GTPase	1.18	1.17	1.57
Receptor-linked protein tyrosine phosphatase (PTP-PS)	1.34	1.56	1.21
Ribosomal protein L13	1.33	1.32	1.17
Secretogranin 3 (Sg3)	1.31	1.36	1.32
Decretogranin II precursor (SGII; SCG2); chromogranin C (CHGC)	1.24	1.46	1.35
Serine/threonine protein phosphatase 2B catalytic subunit alpha; calcineurin A subunit alpha (CALNA); CAM-PRP catalytic subunit; PPP3CA	1.24	1.48	1.67
SHPS-1 receptor-like protein with SH2 binding site	1.17	1.56	1.48
Sodium channel, beta 1 subunit	1.22	1.44	1.46
Sodium/potassium-transporting ATPase beta 1 subunit (ATP1B1)	1.26	1.43	1.65
Sodium/potassium-transporting ATPase beta 2 subunit (ATP1B2)	1.40	1.64	1.32
Synapsin 2A	1.33	1.20	1.23
Synaptosomal associated protein 25; SNAP-25; SNAP; SNAP25; SUP	1.22	1.22	1.65
Tissue carboxypeptidase inhibitor (TCI)	1.11	1.17	1.37
Transducin beta-2 subunit; GTP-binding protein G(i)/G(s)/G(t) beta subunit 2 (GNB2)	1.27	1.44	1.01
Tyrosine phosphatase-like protein; negative regulator of PTPases in neuronal tissues	1.59	1.56	1.42
Vacuolar ATP synthase 16-kDa proteolipid subunit; ATP6C; MVP; ATPL	1.17	1.18	1.32
Vasopressin V2 receptor	1.38	1.04	1.26
Voltage-gated potassium channel protein KV1.1 (RBK1, RCK1, KCNA1)	1.25	1.34	1.21

Differential expression of genes in the rat hippocampus after 3, 7 and 28 days of voluntary wheel running as revealed by microarray analysis. An expression ratio (exercise group : sedentary group) of 1.3–1.8 for at least one time point was considered a 'tendency to change', i.e. indicative of a marginal change for inclusion in this table.

TABLE 2. Hippocampal genes with marginal down-regulation after exercise; the expression ratio (exercise : sedentary group) is presented

Gene alternative names (and common abbreviations)	Expression ratio (exercise : sedentary)		
	3 days	7 days	28 days
14-3-3 protein eta; PKC inhibitor protein-1; KCIP – 1	0.84	0.84	0.84
40S ribosomal protein S29 (RPS29)	0.98	0.85	0.83
Cathepsin L	0.95	0.83	0.76
Dipeptidyl aminopeptidase-related protein (DPP6)	0.67	0.97	0.81
Guanine nucleotide-binding protein G(I) alpha 2 subunit (GNAI2)	0.55	0.50	0.52
HSP84; HSP90-beta; heat shock 90 kDa protein	0.68	0.88	0.78
Id-2; DNA-binding protein inhibitor; HLH protein	0.74	0.62	0.83
Macrophage migration inhibitory factor (MIF)	0.50	0.57	0.67
Mitochondrial ATP synthase D subunit; ATP5H	0.78	0.65	0.68
Protein phosphatase 2A-beta regulatory subunit B (55 kDa); beta -PR55	0.78	0.78	0.78
R-PTP-A; receptor protein-tyrosine phosphatase alpha	0.68	0.57	0.67
ras-related protein m-ras	0.64	0.61	0.64
Tissue inhibitor of metalloproteinase 2 (TIMP2)	0.50	0.60	0.60

Differential expression of genes in the rat hippocampus after 3, 7 and 28 days of voluntary wheel running as revealed by microarray analysis. An expression ratio (exercise group : sedentary group) of 0.5–0.8, for at least one time point, was considered as a 'tendency to change', i.e. indicative of a marginal change for inclusion in this table.

function and are discussed below. Changes in the expression of select genes have been confirmed using Taqman RT-PCR or RNase protection assay (Fig. 2A and B).

Neurotrophic factors

The analysis of the microarray showed that the expression of several neurotrophic factor genes was affected by voluntary exercise with different temporal profiles (Table 3). There was a significant increase in the mRNA levels for the neurotrophins *nerve growth factor* (*NGF*) and *BDNF*, while there were no changes for *neurotrophin-3* (*NT-3*). *NGF* mRNA was elevated only after 3 days of running while *BDNF* mRNA was increased at the three time points examined. Physical activity induced a slight but significant up-regulation of fibroblast growth factor 2 (FGF-2) only at exercise day 3. Microarray analysis showed that the mRNA levels for the BDNF signal transduction *tyrosine kinase receptor* (*Trk*) *B*, were up-regulated after 3 days,

TABLE 3. Genes most significantly changed after exercise; the expression ratio (exercise : sedentary group) is presented

Gene (and common abbreviations)	Expression ratio (exercise : sedentary)		
	3 days	7 days	28 days
Neurotrophic Factors			
Brain-derived neurotrophic factor (BDNF)	2.2*	2.6*	2.4*
Nerve growth factor beta (beta-NGF; NGFB)	2.1*	1.5	1.2
Fibroblast growth factor 2 (FGF2)	1.9*	1.2	1.3
Tyrosine kinase receptor B (TrkB)	1.7	2.1*	1.0
Synapse-related proteins			
Synapsin I (SYN1)	2.1*	2.3*	1.2
Syntaxin 1A (STX1)	2.4*	2.5*	1.9*
Synaptotagmin XI (SYT11)	2.8*	3.2*	3.4*
Neurotransmitter systems			
N-methyl-D-aspartate receptor 2A subunit (NMDAR2A)	1.9*	1.9*	1.3
N-methyl-D-aspartate receptor 2B subunit (NMDAR2B)	2.0*	1.2	1.2
Excitatory amino-acid carrier 1 (EAAC1)	2.4*	1.7	1.2
Gamma-aminobutyric-acid receptor beta 3 subunit ($GABA_A$ beta 3)	0.8	0.3*	1.2
Glutamic acid decarboxylase (GAD65)	1.3	1.1	0.5*
Signal transduction-related molecules			
Calcium/calmodulin-dependent protein kinase type II (CAM kinase II)	3.0*	2.9*	2.5*
Mitogen-activated protein kinase1 (MAP kinase 1)	1.7	2.8*	3.0*
Mitogen-activated protein kinase (MAP kinase 2)	1.6	2.6*	3.1*
Protein kinase C delta (PKC-delta)	1.7	2.0*	1.7
Transcription activators cAMP-responsive element-binding protein (CREB)	1.9*	2.1*	1.3

Differential expression of genes in the rat hippocampus after 3, 7 and 28 days of voluntary wheel running as revealed by microarray analysis. *A gene-expression ratio (exercise group : sedentary group) of $\geqslant$1.8 or $\leqslant$0.5 was considered to reflect a significant difference between the two groups, i.e. the genes were considered to be differentially expressed. For illustrative purpose, these genes have been grouped according to their biochemical function.

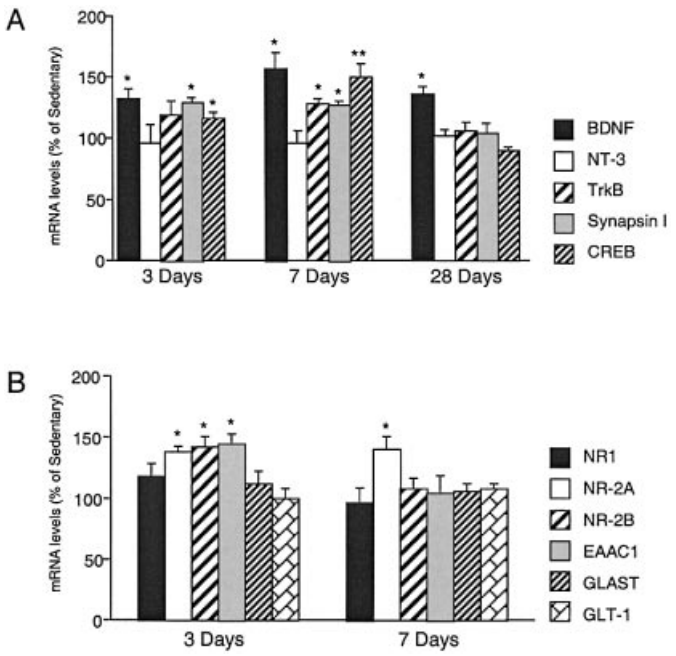

FIG. 2. Effects of 3, 7 and 28 days of voluntary wheel running on genes whose expression was most significantly changed according to array data. mRNA changes were assessed using Taqman RT-PCR (A) and RNase protection assay (B). Each value is expressed as a percentage of sedentary animals (100%), and represents the mean ± SEM of five independent determinations. (*$P < 0.05$).

continued to increase by day 7, and returned to about control levels after 28 days. Taqman RT-PCR confirmed relative levels of *BDNF*, *NT-3* and *TrkB* (Fig. 2A).

Synaptic vesicle trafficking molecules

Among the several proteins involved with vesicular trafficking, the expressions of *syntaxin*, *synaptotagmin* and *synapsin I* were elevated after exercise (Table 3). Microarray analysis showed increased levels of *syntaxin* and *synaptotagmin* in the exercise group at all time points examined. Based on the importance of synapsin I as a downstream effector of BDNF action on the synapse (Jovanovic *et al*., 2000), we quantified mRNA levels of *synapsin I* using Taqman RT-PCR. Taqman RT-PCR confirmed the microarray results, such that the exercise group showed increased levels of *synapsin I* by day 3 as well as by day 7 with respect to control levels (Fig. 2A).

Transcription activators

The expression of CREB, one of the best-characterized transcription factors in the brain that is under the regulatory control of BDNF (Shaywitz & Greenberg, 1999), was elevated after 3 and 7 days of exercise (Table 3). Relative changes in *CREB* mRNA detected using microarray were confirmed using Taqman RT-PCR (Fig. 2A).

Intracellular kinase network member

Microarray showed that several protein kinases involved with modulation of synaptic plasticity were affected by exercise (Table 3). The mRNA levels for *CaM-KII* were elevated at all time points examined. The expressions of *MAP-KI* and *MAP-KII*, implicated in the BDNF action on synaptic plasticity (Grewal *et al*., 1999), were elevated by exercise after day 7. The expression of *PKC*-δ was elevated only after 7 days of exercise.

Neurotransmitter systems

Microarray analysis showed that expression of the glutamatergic NMDA receptor was altered by exercise (Table 3). The mRNA levels for the *NR-2A* and *NR-2B* subunits were increased after 3 days of voluntary wheel running. After 7 days of exercise, the expression of

NR-2B was similar to sedentary animals while the expression of the *NR-2A* subunit remained elevated. An RNase protection assay used to quantify changes in the NMDA receptor subunits showed an increase for *NR-2A* and *NR-2B* mRNA levels after 3 days of exercise (Fig. 2B). Among the glutamate transporters, only EAAC1 was increased after 3 days of exercise. Microarray analysis showed that the mRNA levels for the β_3 subunit of the $GABA_A$ receptor was reduced by 7 days of exercise (Table 3). The expression of *GAD65* was decreased after 28 days of exercise (Table 3).

Discussion

The results emerging from our study indicate that voluntary physical activity modulates the expression of genes associated with neuronal plasticity. We arrived to this conclusion by analysing changes in the relative expression of over 1000 genes that are expressed primarily in the brain. Ninety-four genes showed marginal alterations in expression intensity following exercise, but only genes showing robust alterations were considered significantly changed. This criterion was validated by the measurements of specific mRNAs using quantitative mRNA analysis. Differences in the temporal expression profile among the various gene systems illustrate how acute and chronic exercise can impact the brain at the molecular level. It is notable that BDNF was the only trophic factor gene consistently up-regulated, and that most of the genes affected by exercise have a recognized association with BDNF. It is interesting that the majority of the genes up-regulated by exercise are members of synaptic trafficking machinery or part of signal transduction pathways. These results support a predominant effect of exercise on synaptic function and/or adaptation that is under the spectrum of BDNF action.

Neurotrophic factors

From a pool of 11 trophic factor genes screened, *BDNF*, *NGF* and *FGF-2* were the only genes up-regulated. *NGF* and *FGF-2* genes were up-regulated at exercise day 3, suggesting a role during acute exercise stages (Gomez-Pinilla *et al*., 1997). *BDNF* was the only trophic factor gene that showed a consistent up-regulation at all timepoints examined, confirmed with Taqman RT-PCR. BDNF is expressed predominantly in neurons of the central nervous system, mainly in the hippocampus (Isackson *et al*., 1991). We have previously shown that acute exercise increases *BDNF* mRNA in Ammon's horn 1 and 4 in the hippocampus (Neeper *et al*., 1996). Several studies support the role of BDNF as critical modulator of synaptic plasticity in the hippocampus (Poo, 2001). For example, *BDNF* gene deletion or inhibition (Figurov *et al*., 1996) produces a deficit in long-term potentiation, the transcription-dependent synaptic candidate underlying learning and memory (Nguyen & Kandel, 1996). This deficit in synaptic function can be corrected by exogenous application (Patterson *et al*., 1996) or over-expression (Korte *et al*., 1995) of BDNF. In addiction, as discussed below, various genes associated with the action of BDNF on the synapse were also up-regulated suggesting that elevated BDNF expression resulting from exercise may support synaptic function or plasticity.

Synapse and signal transduction pathways

Exercise increased the expression of several genes associated with synaptic function. In addition to *synapsin I*, exercise increased the mRNAs for *syntaxin* and *synaptotagmin*. It is interesting that *synapsin I* was predominantly up-regulated with short periods of exercise (3 and 7 days), consistent with its role on synaptic vesicle release. *Synaptotagmin* showed a progressive increase with longer periods of exercise, consistent with its role on synaptic vesicle formation (Augustine, 2001). It is known that deletion of the *BDNF* gene in mice results in a reduction of synaptic proteins, fewer docked vesicles and impaired neurotransmitter release (Pozzo-Miller *et al*., 1999). It is well established that BDNF promotes the phosphorylation of synapsin I via activation of TrkB receptors in the presynaptic terminal, resulting in neurotransmitter release (Jovanovic *et al*., 2000). Our results showing parallel increases in *TrkB* and *synapsin I* mRNAs support an action of exercise on the synapse via the BDNF system. It is possible that elevated levels of BDNF as a result of exercise may facilitate synaptic vesicle mobilization and formation, and that prolongation of these events can translate in long-lasting changes in synaptic plasticity.

Exercise up-regulated the genes of several components of the MAP-K cascade such as *MAP-KI* and *MAP-KII*. MAP-K pathways are major downstream signalling cascades of TrkB receptor stimulation (Segal & Greenberg, 1996). MAP-K/ERK is involved in synaptic plasticity and memory formation, and in integration of multiple extracellular signals (Selcher *et al*., 2001; Sweatt, 2001). It appears that MAP-K pathways coordinate several synaptic events in conjunction with CaM-K pathways. For example, synapsin I is phosphorylated by MAP-K and CaM-KII systems (Matsubara *et al*., 1996). It is recognized that CaM-KII affects postsynaptic Ca^{2+} important for synaptic function (Soderling, 2000), and that is involved with hippocampus-dependent memory formation (Fukunaga & Miyamoto, 2000). The expression of *PKC-δ* was significantly increased after 7 days of exercise (Table 3). PKC-δ is required for the activation of MAP-K cascade and for neurite outgrowth (Corbit *et al*., 1999). Members of the CaM-K family showed the largest increase with short exercise periods while members of the MAP-K pathway showed a progressive increase with longer exercise time (after 7 days). The difference in the temporal profile between the activation of these two signalling systems may be associated to the differential effects of acute vs. chronic exercise.

Neurotransmitter systems

Exercise up-regulated *CaM-KII* and *NMDA* receptor with a similar temporal profile, which is consistent with studies showing that NMDA receptor function is susceptible to regulation via interactions with CaM-KII cascade (Scheetz *et al*., 2000). Short-term exercise increased the expression of specific subunits, e.g. *NR2A* and *NR2B*, of the NMDA receptor system as detected with microarray and confirmed using RNase protection assay. Changes in *NR2B* occurred at 3 days while *NR2A* was still up-regulated at day 7. NR2A is expressed in the majority of synapses in the mature brain (Monyer *et al*., 1994), and plays a major role in synaptic plasticity modulating long-term potentiation and depression (Sprengel *et al*., 1998). BDNF and NMDA receptors have reciprocal effects on the synapse (Black, 1999). Acute, *in vitro* exposure of cortical neurons to BDNF rapidly enhances NMDA receptor activity by increasing channel open probability through the phosphorylation of the NR2B subunits (Levine & Kolb, 2000).

The glutamate transporter, *EAAC1*, was up-regulated by 3 days only, detected with microarray (Table 3) and confirmed with RNase protection assay (Fig. 2B). EAAC1 is one of the five isoforms of the glutamate transporter responsible for the removal of extracellular glutamate from the synaptic cleft (Gadea & Lopez-Colome′, 2001). As excessive release of glutamate can cause neuronal death (Rossi *et al*., 2000), the increased expression of *EAAC1* observed may represent a protective mechanism activated by exercise.

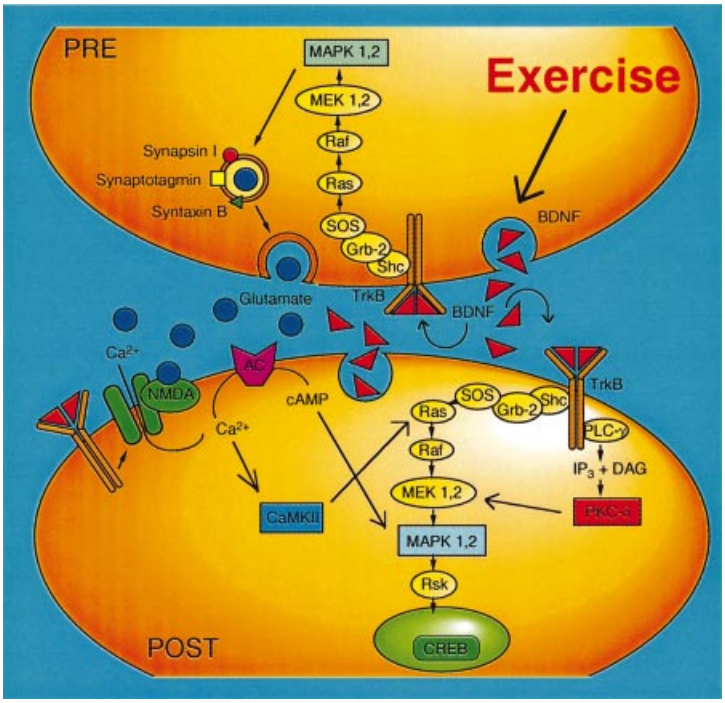

FIG. 3. Potential mechanisms by which exercise can modulate neuronal plasticity in the hippocampus. Elevated expression of BDNF resulting from exercise can affect neuronal plasticity acting at both pre- and postsynaptic terminals. Signal transduction of BDNF is mediated primarily through the TrkB receptor, whose expression was up-regulated by exercise. TrkB signalling at the pre or postsynaptic terminals results in up-regulation of several downstream genes such as *MAP-KI* and *MAP-KII*, *PKC-δ* and *CaM-KII*. Moreover, at the presynaptic terminals, exercise can act on synapsin, synaptotagmin andsyntaxin to modulate neurotransmitter release. Additionally, the postsynaptic effect of exercise could be mediated through Ca^{2+} influx via the NMDA receptor, whose expression was up-regulated by exercise. This in turn can activate the MAP-K cascade via CaM-K. The activated MAP-K may act on a nuclear target, such as the transcription factor CREB, which was also up-regulated by exercise.

Microarray analysis showed reduced mRNA levels for specific components of the GABAergic system. The $GABA_A$-β_3 subunit, a main component of the GABAA receptor in hippocampus (Rudolph *et al*., 2001), was reduced after 3 and 7 days of exercise. Moreover, the expression of the enzyme responsible for the GABA synthesis, GAD65, was reduced after 28 days of voluntary running. GAD65 has also been involved with the neuronal GABA release (Esclapez *et al*., 1994). It has been shown that GABA function inhibition can enhance functional recovery after CNS injury (Raineteau & Schwab, 2001). It is possible therefore that exercise can benefit functional recovery by reducing GABA function (Norman *et al*., 1998). Moreover, the fact that GABA reduces BDNF expression in the hippocampus (Zafra *et al*., 1991), suggests that exercise may elevate levels of BDNF by reducing GABA function.

Transcription factors

Exercise elevated the expression of the transcription factor CREB, as detected using microarray and Taqman RT-PCR. CREB can regulate BDNF gene transcription in a calcium-dependent mechanism (Finkbeiner, 2000a, b). In turn, through the MAP-K cascade, BDNF causes the phosphorylation of CREB resulting in CREB activation and gene transcription (Finkbeiner *et al*., 1997). CREB is required for various forms of memory (Silva *et al*., 1998), and appears to play a role in neuronal resistance to insult (Walton *et al*., 1999). Hippocampal slices from mice with reduced levels of CREB show impairment in the maintenance of long-term potentiation (Bourtchuladze *et al*., 1994), a postulated mechanism for certain forms of learning. The largest increase in *CREB* mRNA was observed after 7 days of exercise, which is consistent with the induction of the MAP-K members.

How exercise can affect neuronal plasticity

Our results show specific molecular systems through which exercise may impact neural function (Fig. 3). Genes primarily up-regulated are members of synaptic vesicle trafficking machinery or part of secondary messenger pathways, whose activation may affect synaptic function. Most of the genes affected by exercise have a recognized association with BDNF, supporting a central role of BDNF on the mechanisms by which exercise translates into neural changes. *TrkB* and *synapsin I* genes are up-regulated at early phases and these changes may result in immediate early events such as synaptic vesicle release. General intracellular signal pathways, such as MAP-K/ERK and CaM-K seem to be involved but at different periods of the exercise training. According to our results, CaM-K pathways are highly up-regulated with short-term exercise while MAP-K pathways seem to become more important gradually, when exercise extends for longer periods. CaM-K is closely regulated by the NMDA receptor system, which responded to short-term exercise. According to this view, the elevated expression of the NMDA-R subunits resulting from exercise could represent a downstream effect of exercise during acute phases. Probably, along the course when exercise becomes more chronic, other types of events take place which would result in more permanent cellular changes. For example, MAP-K signalling and synaptotagmin function may be associated with long-term effects

of exercise. Indeed, synaptotagmin has been associated with synapse vesicle formation that may result in more long lasting synaptic events.

Our results have been obtained in the hippocampus, which is deeply involved with learning and memory. It is important to establish whether events described for the hippocampus can also apply to other brain regions. Present results showing specific molecular systems modulated by exercise provide a framework to guide future studies to further dissect the mechanisms by which exercise translates into changes of neuronal plasticity and function.

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Abbreviation

BDNF, brain-derived neurotrophic factor; CaM-KII, Ca^{2+}/calmodulin-dependent protein kinase cDNA, complementary DNA; CNS, central nervous system; CREB, cyclic AMP response element binding protein; EAAC1, excitatory amino acid carrier 1; GABA, gamma-aminobutyric acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP-K/ERK, mitogen-activated/extracellular signal-regulated protein mRNA, messenger RNA; NMDAR, *N*-methyl-D-aspartate receptor; PKC, protein kinase C; RT-PCR, Real-time quantitative reverse transcription polymerase chain reaction; TrkB, tyrosine kinase receptor B.

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