ORIGINAL

Gene-expression profile reveals the genetic and acquired phenotypes of hyperactive mutant SPORTS rat

Taigo Horiguchi[1], Yumiko Miyatake[2,†], Keiko Miyoshi[1], Ayako Tanimura[1,††], Hiroko Hagita[1], Hiroshi Sakaue[2], and Takafumi Noma[1]

[1]Department of Molecular Biology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto, Tokushima 770-8504, Japan, [2]Department of Nutrition and Metabolism, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Present address, [†]Department of Physiology, Wakayama Medical University School of Medicine, 811-1, Kimiidera, Wakayama City, Wakayama 641-8509, Japan, [††]Division of Food & Health Environmental Sciences, Department of Environmental & Symbiotic Sciences, Faculty of Environmental & Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100, Tsukide, Higashi-ku, Kumamoto 862-8502, Japan

Abstract : Spontaneously Running Tokushima Shikoku (SPORTS) rat is a hyperactive rat strain. However, the causative mutation of this phenotype has not yet been identified. To investigate the molecular basis for the unique phenotype of SPORTS rats, we examined gene-expression profiles by microarray analyses. Among adenylate kinase isozymes that maintain the homeostasis of cellular adenine nucleotide composition in the cell, only adenylate kinase 1 is highly up-regulated in both exercised and sedentary SPORTS rats compared with wild-type (WT) rats, 5.5-fold and 3.3-fold, respectively. Further comparative analyses revealed that genes involved in glucose metabolism were up-regulated in skeletal muscle tissue of exercised SPORTS rats compared with sedentary mutants, whereas genes related to extracellular matrix or region were down-regulated compared with WT rats. In brain tissue of sedentary SPORTS rats, genes associated with defense and catecholamine metabolism were highly expressed compared with WT rats. These findings suggest that genetic mutation(s) in SPORTS rat remodels metabolic demands through differentially regulating gene expression regardless of exercise. Therefore, the SPORTS rats are useful animal model not only for further examining the effects of exercise on metabolism but also for deeply studying the molecular basis how mutation affect the psychological motivation with spontaneous voluntary exercise phenotype. J. Med. Invest. 67 : 51-61, February, 2020

Keywords **:** *adenylate kinase, energy metabolism, microarray analysis, SPORTS rats, voluntary exercise*

INTRODUCTION

Daily exercise is known to have positive effects on maintaining physical and mental health, including the prevention of metabolic syndrome (1) and locomotive syndrome (2). It is also known to improve mental health (3), cardiopulmonary function (4), and immune function (5). To understand the molecular mechanism of these effects of exercise, several genomic and transcriptomic approaches through human and mouse model studies have been attempted (1-5). However, the precise mechanistic role of exercise in human health has not been fully established because of the complexity of genomic and epigenomic regulation (6).

We previously reported a novel voluntary exercise rat strain, the Spontaneously Running Tokushima Shikoku (SPORTS) rat (7). The SPORTS rat was spontaneously isolated as a hyperactive mutant of Wistar rats and established as an inbred line. They have various features, such as lighter weight, lower body fat, and lower serum insulin level than wild type rats. In particular, male SPORTS rats show hyper running activity. They run six times longer than WT rats, whereas female SPORTS rats do not run for such long distances compared to males. In females, running distance depends on the estrus cycle. Only male offspring display excessive running activity, suggesting that the hyperactive phenotype is genetically and hormonally controlled. After 4 weeks of running, body weight and abdominal fat were lower in male SPORTS rats than in WT rats. Serum insulin levels were also decreased in male SPORTS rats (7), suggesting an improved state of glucose and lipid metabolism because of exercise. However, the SPORTS rat strain has a tendency to form left atrial thrombosis (8), the reasons for which are currently unknown.

Extracellular norepinephrine (NE) levels in the hippocampus of SPORTS rats with or without exercise using running wheels were higher than those of WT rats, suggesting that high concentration of NE in the hippocampus is caused by genetic background, but not by simple running exercise (9). One possible reason for high NE levels was the lower activity of monoamine oxidase A (MAOA), which degrades NE in the hippocampus, as evidenced by the fact that the protein expression level of MAOA in SPORTS rats was 40% lower than that in WT rats, and that its activity was significantly lower at 4 weeks (9). In addition, we found the following three notable characteristics of the SPORTS rat strain. First, injection of adiponectin into the lateral ventricle of SPORTS rats decreased home cage activity (10). Second, plasma levels of des-acyl ghrelin were lower in SPORTS rats than in WT rats, and injection of ghrelin inhibitor into WT rats promoted voluntary activity. Third, when ghrelin was intraperitoneally injected into SPORTS rats, running wheel activity was decreased (11). These results suggested that signals secreted from tissues such as fat tissue and stomach may regulate voluntary exercise.

In this study, to understand the mechanistic link between genetic change(s) and phenotypes of the SPORTS rat strain, we

Received for publication July 1, 2019 ; accepted August 25, 2019.

Address correspondence to Takafumi Noma, M.D., Ph.D., Department of Molecular Biology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15, Kuramoto, Tokushima 770-8504, Japan and Fax : +81-88-633-7326.

analyzed gene-expression profiles among hyperactive SPORTS rats, sedentary SPORTS rats, and control WT rats. We found several unique characteristics of gene expression in the skeletal muscle and brain tissue caused by either genetic or acquired change(s).

MATERIALS & METHODS

Animals

SPORTS rats were housed in individual cages with or without running wheels, at constant room temperature (23°C ± 1°C) with a 12-h light-dark cycle, and were fed *ad libitum*. Exercised SPORTS rats were kept in an environment capable of continuously free accessing the wheel running from the time of 4 weeks of age until being subjected to dissection (15-week-old). Rats were anesthetized with diethyl ether, then euthanized by cervical dislocation to isolate tissues for western blotting and microarray analyses. Male Wistar rats (15-week-old) were purchased from Japan SLC Co. (Shizuoka, Japan) and used as controls. All procedures were conducted according to the guidelines for the care and use of laboratory animals of Tokushima University Graduate School (Certification No. 13070).

Western blotting

Tissues (cerebrum, cerebellum, heart, liver, kidney, red muscle, and white muscle) were excised from 15-week-old male SPORTS and WT rats (three individuals each), homogenized with a Physcotron homogenizer (Microtec Co., Ltd., Chiba, Japan) in extraction buffer (62.5 mM Tris–HCl, pH 6.8 ; 2% SDS), and sonicated with a Sonifier 250 (Branson Ultrasonics, Danbury, CT, USA). After centrifugation, supernatants were collected as samples for Western blotting analyses. Western blotting and immune detection were performed following the standard method using 10 or 20 μg proteins for C2C12 or tissue samples, respectively. Anti-adenylate kinase-specific antibodies used were developed in our laboratory (12, 13), and anti-GAPDH antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Obtained signal was quantified using an imaging system, ChemiDoc XRS (Bio-Rad, Hercules, CA, USA) and a software (Image Lab ; Bio-Rad). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Microarray

Hindlimb skeletal muscle and brain tissue (cerebrum) were excised from SPORTS rats, sedentary SPORTS rats, and WT rats (male, 15-week-old), respectively. Total RNA was isolated from these tissues and purified using RNeasy Mini Kit columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After confirming the quality and quantity of prepared RNA, microarray analyses were performed using a Whole Rat Genome Oligo DNA Array (4 × 44K, Agilent) for skeletal muscle tissue and SurePrint G3 (8 × 60K, Agilent) for brain tissue.

Data obtained from skeletal muscle tissue were normalized and analyzed with GeneSpring software (v. 13.0, Agilent Technologies, Santa Clara, CA, USA). The percentile target was set to 75 and the baseline was adjusted to median to all samples. Data for brain tissue were normalized using the global scaling method (Takara Bio Inc., Shiga, Japan). The trimmed mean probe intensity was determined by removing 2% of the lower and higher ends of the probe intensities for calculating the scaling factor. Normalized signal intensities were then calculated from the target intensity on each array using the scaling factor such that the trimmed mean target intensity of each array was arbitrarily set to 2500.

For microarray analysis of skeletal muscle tissue, we used one rat from each rat type. To confirm the microarray results, the expression levels of several selected genes were validated by RT-PCR (data not shown). For microarray analysis of brain tissues, we used three rats from each rat type, and the average signal strength of each gene was used for comparison of sedentary SPORTS rats with WT rats. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) and are accessible through GEO series accession numbers GSE87762 for brain tissue and GSE88853 for skeletal muscle tissue.

Enrichment analyses of microarray data were performed using DAVID 6.7 (The Database for Annotation, Visualization and Integrated Discovery ; http://david.ncifcrf.gov) (14, 15). For enrichment analyses of up-regulated genes of muscle, the gene probes whose expression levels were > 2 fold higher in the sample than in the comparison control were selected. Then the probes flagged "Compromised" either in the sample or in the comparison control or both were eliminated from the list, and the probes flagged "Not detected" in the sample were also omitted. The rest of gene probes were used for enrichment analyses with DAVID. In the case of analyses of down-regulated genes, probes for analyses were selected similarly. The gene probes whose expression levels were < 2 fold lower in the sample than in the comparison control were selected. Then the probes flagged "Compromised" and "Not detected" were eliminated. For enrichment analyses of up-regulated genes of brain, the gene probes whose average expression levels were > 2 fold higher in the sample than in the comparison control were selected. Then the probes flagged unreliable in two of three samples were eliminated. The rest of gene probes were used for enrichment analyses with DAVID. In the case of analyses of down-regulated genes, the probes whose average expression levels were < 2 fold lower were selected similarly. Pathway analyses were performed using KEGG (http://www.genome.jp/kegg/) and WikiPathways (http://wikipathways.org/index.php/WikiPathways) information. Investigation of transcription factors was performed using a Genomatix genome analyzer (Genomatix Software GmbH, Munich, Germany).

RESULTS

Expression of adenylate kinase isozymes

Because SPORTS rats exhibit excessive running activity, we first assessed the change in gene expression related to energy metabolism. Adenylate kinase (AK) isozymes, which catalyze a reversible high-energy phosphoryl transfer reaction between adenine nucleotides, are the key enzymes of energy homeostasis (16). Thus, we examined the protein expression levels of AK isozymes in various tissues (cerebrum, cerebellum, heart, liver, kidney, red skeletal muscle, and white skeletal muscle) of exercised SPORTS rats, sedentary SPORTS rats, and WT rats by Western blotting (Fig. 1A). AK1 expression was detected in all examined tissues except the liver, and the expression level was significantly higher in skeletal muscle and brain tissue of SPORTS rats than in WT rats regardless of exercise (Fig. 1A, 1B). High AK2 expression was detected in the heart, liver, kidney, and AK3 expression was observed strongly in the heart, liver, and kidney, but weakly in brain and skeletal muscle tissue. Expression of AK4 was detected in all tissues examined except skeletal muscle tissue (Fig. 1A). There were no gross differences in AK2, AK3, and AK4 expression levels between SPORTS rats and WT rats, consistent with previous reports (Fig. 1B) (13, 17,18).

Enrichment analyses of microarray data of skeletal muscle

To further analyze genome-wide gene expression in SPORTS rats, we performed microarray analysis using RNA isolated from

Fig 1A

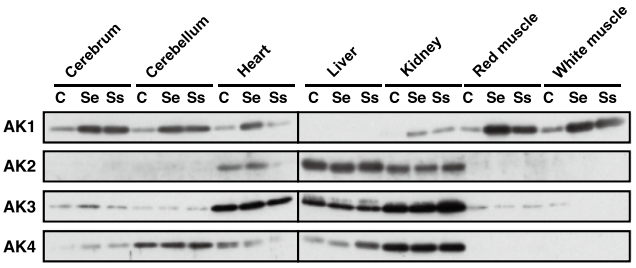


Fig 1B

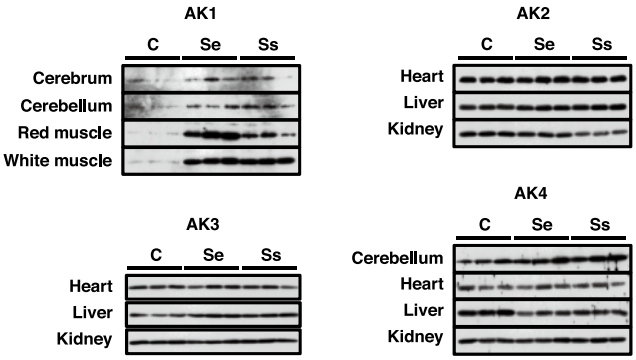


Fig 1. Western blot analysis of adenylate kinase isozymes
(A) Protein expressions of adenylate kinase isozymes (AK1, AK2, AK3, and AK4) in various tissues (cerebrum, cerebellum, heart, liver, kidney, red skeletal muscle, and white skeletal muscle) were examined by Western blot analysis using each isozyme-specific antibodies. Twenty micrograms of protein was applied to each lane. (B) Protein samples from three rats each (one is the same as Fig.1A) of exercised SPORTS rats, sedentary SPORTS rats, and sedentary wild-type rats were analyzed by Western blotting. Se, exercised SPORTS rat; Ss, sedentary SPORTS rat; C, sedentary wild-type rat.

skeletal muscle tissue of three groups, categorized into exercised SPORTS rat, sedentary SPORTS rat, and WT Wistar rat.

Overall, 1,687 probes were up-regulated > 2 fold and 2,302 probes were down-regulated < 0.5 fold in exercised SPORTS rats compared to sedentary SPORTS rats. On the other hand, 754 probes were up-regulated > 2 fold and 391 probes were down-regulated < 0.5 fold in sedentary SPORTS rats compared to WT rats. *Ak1* expression levels in both exercised and sedentary SPORTS rats were higher than that in WT rats, whereas the *Ak2*, *Ak3*, and *Ak4* expression levels were almost similar between SPORTS rats and WT rats (Table 1). These results are consistent with Western blot results for the AK isozymes.

Then, we performed GO analysis by DAVID algorithm (14, 15). Compared to sedentary SPORTS rat, exercised SPORTS rat showed 224 up-regulated clusters overall, and the top 10 clusters with the highest enrichment scores ; this shows the over-representation of gene-based association in biological processes (Table 2A). Among them, *muscle contraction*-related and *glucose metabolism*-related genes were top-ranked. DAVID analysis of the down-regulated genes in exercised SPORTS rats showed that *extracellular matrix*-related and *defense response*-related genes were highly enriched compared to sedentary SPORTS rat (Table 2B). Furthermore, comparative analyses between sedentary SPORTS rats and WT rats revealed that *extracellular region*-related genes were positioned at the top of the enrichment score and that *immune response*-related genes were positioned second among up-regulated genes, as shown in Table 3A. The clusters

Table 1. Comparison of the expression levels of *Ak* isozymes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Ak1	5.53	3.27	2.28
Ak2	1.29	0.72	1.12
Ak3	0.89	0.77	1.09
Ak4	0.97	0.75	1.12

The comparative expression levels of *Ak* isozymes of exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats.

Table 2A. Enrichment analyses of gene expression in skeletal muscle: exercised SPORTS rat vs. sedentary SPORTS rat

Cluster	Term	Enrichment score
1	Contractile fiber	6.44
2	Muscle contraction	6.15
3	Glucose metabolic process	4.56
4	Muscle organ development	3.62
5	Basic leucine zipper	3.49
6	Response to hormone stimulus	3.41
7	Response to glucose stimulus	3.35
8	Calmodulin binding	2.80
9	Insoluble fraction	2.71
10	Glycogen metabolism	2.69

Table 2B. Enrichment analyses of gene expression in skeletal muscle: exercised SPORTS rat vs. sedentary SPORTS rat

Cluster	Term	Enrichment score
1	Extracellular region	14.91
2	Extracellular matrix	14.79
3	Polysaccharide binding	13.07
4	Actin binding	9.13
5	Defense response	8.63
6	Cell adhesion	7.75
7	Response to hormone stimulus	5.86
8	Immune response	5.64
9	Blood vessel development	5.38
10	Developmental growth	5.07

Gene expression levels in skeletal muscle tissue of exercised SPORTS rat and sedentary SPORTS rat were comparatively analyzed by Functional Annotation Tool of DAVID. (A) Analyses of up-regulated genes (> 2-fold), and (B) down-regulated genes (< 2-fold) in exercised SPORTS rat compared to sedentary SPORTS rat. Obtained top 10 clusters, their representative terms of biological function, and enrichment scores are listed.

of down-regulated genes generated by DAVID analysis were relatively classified among the low enrichment scores. However, *cell adhesion*, *response to hormone stimulus*, *extracellular matrix* cluster groups were listed among the highest enrichment scores (Table 3B). These results suggest that gene expression in skeletal muscle tissue is regulated by both genetic mutation and exercise.

Table 3A. Enrichment analyses of genes in skeletal muscle: sedentary SPORTS rat vs. sedentary WT rat

Cluster	Term	Enrichment score
1	Extracellular region	4.86
2	Immune response	4.21
3	Extracellular matrix	3.49
4	Response to lipopolysaccharide	3.27
5	Polysaccharide binding	2.87
6	Chemokine activity	2.72
7	Scaveneger receptor activity	2.60
8	Peptidase activity	2.36
9	Reulation of inflammatory response	2.16
10	Antigen processing and presentation	2.00

Table 3B. Enrichment analyses of genes in skeletal muscle: sedentary SPORTS rat vs. sedentary WT rat

Cluster	Term	Enrichment score
1	Cell adhesion	2.38
2	Responset to hormone stimulus	2.16
3	Extracellular matrix	2.07
4	Extracellular region	2.03
5	Muscle contraction	2.02
6	Ion channel activity	1.73
7	Oxygen transport	1.69
8	Carboxylic acid binding	1.60
9	ATPase activity	1.39
10	PAS	1.24

Gene expression levels in skeletal muscle tissue of sedentary SPORTS rat and sedentary WT rat were comparatively analyzed by Functional Annotation Tool of DAVID. (A) Analyses of up-regulated genes (> 2-fold), and (B) down-regulated genes (< 2-fold) in sedentary SPORTS rat compared to sedentary WT rat. Obtained top 10 clusters, their representative terms of biological function, and enrichment scores are listed.

Pathway analysis of glycolysis-related genes

Because the enrichment score of the clusters related to *glucose metabolism* was relatively high in exercised SPORTS rats compared to sedentary SPORTS rats, we next compared expression levels of all genes in glycolysis/gluconeogenesis pathways and plotted a pathway map (Table 4, Fig. 2). The result clearly demonstrated that many of glycolysis-related genes were up-regulated by exercise. In contrast, the expression of TCA cycle-related genes was maintained at a similar level between exercised SPORTS rats and sedentary SPORTS rats (Table 5). Up-regulation of pyruvate dehydrogenase kinase isozymes (*Pdk2* and *Pdk4*, Table 6), which are PDH inhibitors, is consistent with a preference for glycolysis.

Table 4. Comparison of the expression levels of glycolysis-related genes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Hk1	1.11	0.75	0.98
Hk2	1.70	1.05	1.08
Gpi	0.80	4.72	0.92
Pfkm	0.80	4.87	1.00
Aldoa	0.75	4.09	0.90
Tpi1	0.84	2.87	0.98
Gapdh	0.98	2.32	0.98
Pgk1	0.89	2.66	1.02
Pgam2	0.92	3.10	0.72
Eno1	0.89	0.85	0.97
Pkm2	0.91	4.69	—†

Comparison of the expression levels of glycolysis-related genes in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†," this gene did not appear in the list.

Table 5. Comparison of the expression levels of TCA cycle-related genes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Cs	1.04	0.91	0.93
Aco1	0.76	0.96	1.02
Idh1	0.39	0.67	0.85
Ogdh1	—†	—†	0.94
Dlst	0.92	0.89	0.96
Suclg1	1.03	1.27	1.13
Sdha	0.92	1.00	1.05
Fh1	1.17	0.96	1.22
Mdh1	1.15	0.54	0.94

Comparison of the expression levels of TCA cycle-related genes in exercised SPORTS rat, sedentary SPORTS rat, and WT rat (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†" indicates those cases in which the values of both samples were too small to compare.

Table 6. Comparison of the expression levels of Pdk isozyme genes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Pdk1	1.18	0.26	0.92
Pdk2	1.06	1.34	1.03
Pdk3	—‡	—§	0.90
Pdk4	0.53	4.71	—†

Comparison of the expression levels of Pdk isozyme genes in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†" values of both Ss and C were too small to compare; "‡" value of C was too small to compare; and "§" value of Se was too small to compare.

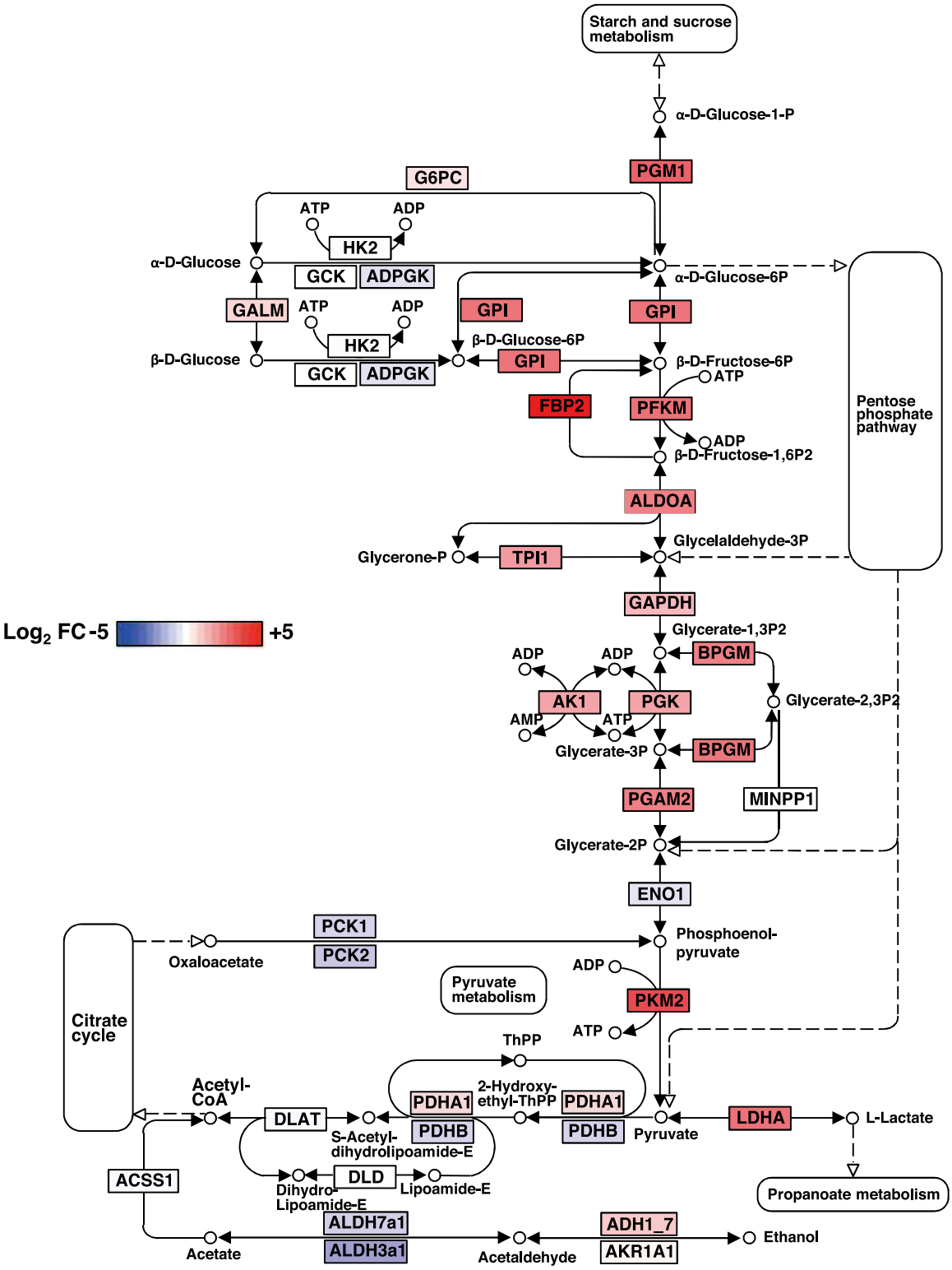
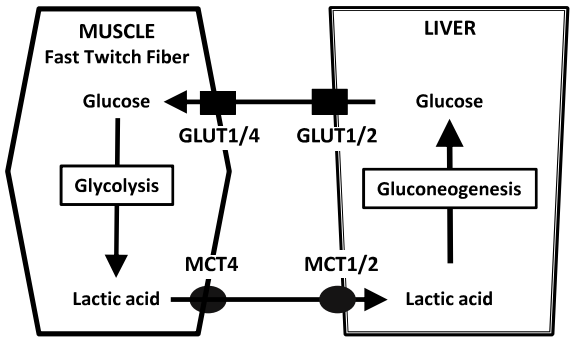


Fig 2. Gene expression of glycolysis/gluconeogenesis-related genes
Comparison of gene expression in the glycolytic pathway between exercised SPORTS rat and sedentary SPORTS rat is depicted using KEGG pathway map. In exercised SPORTS rat, up-regulated genes are colored in red and down-regulated genes in blue.

Analysis of Mct expression levels to investigate lactate dynamics

In addition to glycolytic genes, the expression of *Ldha* gene, which catalyzes lactate formation from pyruvate (Fig. 2), was up-regulated 4.86-fold by exercise. This finding implied excess lactate production in skeletal muscle tissue of exercised SPORTS rat and prompted us to analyze monocarboxylate transporter (MCT) isozyme expression. Among MCT isozymes, MCT4 mediates lactate efflux, whereas MCT1 and MCT2 mediate its influx (19) (Fig. 3). *Mct1* expression levels were not grossly changed between sedentary SPORTS rats and WT rats (1.19 fold), whereas *Mct1* expression in exercised SPORTS rats was strongly down-regulated compared to sedentary SPORTS rats (0.40 fold). *Mct4* expression in sedentary SPORTS rat was slightly lower than that in WT rat (0.69 fold), whereas *Mct4* expression in exercised SPORTS rat was remarkably up-regulated compared to sedentary SPORTS rats (10.55 fold). Although *Hif1a* is an important regulator of *Mct4* gene expression (20), *Hif1a* expression in exercised SPORTS rats was not elevated compared to sedentary SPORTS rat (0.78 fold). These findings suggest the metabolic adaptation to the higher energy demand caused by exercise, but not genetic change(s) such as oncogenic changes through *Hif1a* up-regulation.

A



B

Gene	Fold change	
	Se vs C	Ss vs C
Mct1	0.40	1.19
Mct4	10.55	0.69

Fig 3. Gene expression of Mct isoform genes
(A) Relationships between MCTs and glucose metabolism are illustrated. (B) *Mct1* and *Mct4* expression levels were compared among three different types of each rat. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rat ; C, sedentary wild-type rat.

Enrichment analyses of microarray data of brain tissue

To gain insight into genetic background of SPORTS rats, we also performed microarray analysis using brain tissue of sedentary SPORTS rats and WT rats. Overall, 772 probes were up-regulated (> 2 fold) and 636 probes were down-regulated (< 0.5 fold) in sedentary SPORTS rats compared to WT rats. Cluster analysis of microarray data of brain showed no gross difference in the gene expression patterns between sedentary SPORTS rat and wild-type rat. Enrichment analysis of the up-regulated genes showed that the cluster groups *extracellular region, defense response, sexual reproduction*, and *catecholamine metabolism* were highly enriched. The same analysis of down-regulated genes showed that the cluster groups *hormone activity, response to ethanol*, and *extracellular region* were obtained with relative high enrichment scores (Table 7A, 7B). These results suggest that brain-specific gene expression in sedentary SPORTS rats was also affected by the genetic mutation.

Table 7A. Enrichment analysis of genes in brain tissue: sedentary SPORTS rats vs. sedentary WT rats

Cluster	Term	Enrichment score
1	Extracellular region	5.00
2	Defense response	3.87
3	Sexual reproduction	3.23
4	Catecholamine metabolic process	2.79
5	Serotonin metabolic process	2.47
6	Polysaccharide binding	2.39
7	Extracellular matrix	2.30
8	Neuron maturation	1.99
9	Intrinsic to plasma membrane	1.92
10	Response to hormone stimulus	1.79

Table 7B. Enrichment analysis of genes in brain tissue: sedentary SPORTS rats vs. sedentary WT rats

Cluster	Term	Enrichment score
1	Hormine activirt	4.83
2	Response to ethanol	3.86
3	Extracellular region	3.78
4	Neuropeptide signaling pathway	3.40
5	Muscle contraction	3.37
6	Response to hormone stimulus	2.56
7	Reproductive behavior	2.32
8	Circadian behavior	2.29
9	Intrinsic to membrane	2.00
10	Serotonin binding	1.98

Gene expression levels in brain tissue of sedentary SPORTS rats and sedentary WT rats were comparatively analyzed by Functional Annotation Tool of DAVID. (A) Analyses of up-regulated genes (> 2-fold), and (B) down-regulated genes (< 2-fold) in sedentary SPORTS rats compared to sedentary WT rats. Obtained top 10 clusters, their representative terms of biological function, and enrichment scores are listed.

DISCUSSION

Because of its voluntary hyperactivity, we hypothesized that the SPORTS rat strain has specific properties related to energetic and psychological metabolism. To investigate the causative mechanisms of voluntary activity and the metabolic properties, we performed comparative microarray analyses and subsequent *in silico* analyses of gene-expression profiles. The possible genotype-phenotype linkage in SPORTS rats was summarized in Figure 5. Genetic mutation in SPORTS rat may regulate the motivation and the high-energy demand. The high energy demand may be supported by not only activation of glycolysis but also high turnover of adenine nucleotide cycle through AK1 up-regulation, resulting in metabolic adaptation, in skeletal muscle and brain. We discuss as following.

Effects of exercise on gene expression

KEGG pathway analysis using microarray data revealed that the glycolytic pathway was enhanced in muscle of exercised SPORTS rat. This enhancement seems to be the effect of exercise on energy metabolism, suggesting the energetic adaptation through the metabolic remodeling of muscle cells in response to exercise.

Microarray data also showed that *Ak1* expression was enhanced in skeletal muscle by exercise in SPORTS rats (Figure 1A). In the glycolytic pathway, ATP or ADP is required for overcoming three rate-limiting steps (Figure 2), and therefore cytosolic AK1 might promote their balance through interconversion. Previous *Ak1* knockout study demonstrated the increased glycolytic gene expression, suggesting a close relationship between AK1 and glycolysis (21). In the case of SPORTS rats, sedentary rat itself showed higher level of *Ak1* expression and exercised rat exhibited further increased *Ak1* expression. Therefore, there may be at least two independent regulatory mechanisms of *Ak1* gene expression such as genetic mutation and adaptation. Moreover, among *Ak* isozyme genes, only *Ak1* was up-regulated in SPORTS rats, indicating that Ak1 expression is controlled by high turnover of high energy phosphoryl transfer in cytoplasm but not in mitochondria like other Ak isozymes.

PDK4 is a repressor of pyruvate dehydrogenase (PDH) and is specifically expressed in heart and skeletal muscle tissues (22). It has been reported that the transcription of *Pdk4* is increased during prolonged exercise under the control of *Foxo1* (23). In agreement with previous reports, our microarray analysis also showed that *Pdk4* was specifically up-regulated in skeletal muscle tissue of exercised SPORTS rats (Table 6). PDH catalyzes acetyl CoA formation from pyruvate ; thus repression of *Pdh* implies that metabolic flux from glycolysis to TCA cycle is blocked in skeletal muscle tissue of exercised SPORTS rats. On the other hand, expression of TCA cycle-related genes did not differ greatly between exercised SPORTS rat and sedentary SPORTS rat (Table 5). These results suggest that glycolysis is preferentially used to satisfy the energy demand of exercised SPORTS rats.

Among clusters listed in Table 2A (genes up-regulated with exercise), *muscle contraction*-related, *muscle development*-related, and *glucose metabolism*-related clusters showed higher enrichment scores. These clusters contain many genes of fast-twitch fiber components, such as *Myh1* (89.72-fold), *Myh2* (10.20-fold), *Myh4* (2330.69-fold), *Myl1* (2.04-fold), *Tnni2* (14.13-fold), and *Tnnt3* (24.82-fold) (Table 8). On the other hand, genes of slow-twitch fiber components, such as *Myh7* (0.22-fold), *Myl2* (0.06-fold), *Myl3* (0.26-fold), *Tnni1* (0.09-fold), and *Tnnt1* (0.39-fold), were all down-regulated with exercise. From these results, slow-to-fast fiber type transition seems to be taken place in skeletal muscle tissue of exercised SPORTS rats. Of interest, the expression levels of these fast-twitch fiber genes in sedentary SPORTS rat were lower than in wild-type (WT) rat (Table 8). These findings suggest that genetic mutations in SPORTS rats affect the transcriptional regulation of fast-twitch fiber genes, though we could not determine the responsible transcription factor(s) at this moment.

Fast-twitch fiber depends on glycolysis to meet its energy demand (24). Thus, the up-regulation of fast-twitch fiber genes is consistent with the up-regulation of glycolytic genes in skeletal muscle tissue of exercised SPORTS rats. Because *Ak1* is highly expressed in fast-twitch fiber (21), the up-regulation of fast-twitch fiber genes is also paralleled with the *Ak1* gene up-regulation in skeletal muscle tissue of exercised SPORTS rats.

In addition, microarray analyses of SPORTS rats revealed that the expression levels of myokines such as *Myostatin*, *Il4*, *Il5*, *Il6*, *Il7*, and *Bdnf* were very low in all tested rats. Among the myokines, only *Il15* was up-regulated (12.43-fold) in exercised SPORTS rats compared to sedentary SPORTS rat (Table 9). *Il15* was originally identified as a T-cell growth factor (25, 26), and is now known to be one of cytokines that is expressed in various cells and that exerts a pleiotropic function in various tissues (27).

Table 8. Comparison of the expression levels of muscle fiber-related genes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Myh1	0.10	89.72	0.56
Myh2	0.05	10.20	0.89
Myh4	0.07	2330.69	—†
Myh7	0.95	0.22	0.54
Myl1	0.85	0.06	—†
Myl2	0.95	0.06	—†
Myl3	0.98	0.26	0.76
Tnni1	0.98	0.09	—†
Tnni2	0.10	14.13	1.62
Tnnt1	0.99	0.39	0.30
Tnnt3	0.09	24.82	—†

Comparison of the expression levels of skeletal muscle fiber-related genes in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†," values of all samples were too small to compare.

Table 9. Comparison of the expression levels of myokines

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Il15	0.82	12.43	0.70
Bdnf	—†	—†	1.05
Ucp3	0.53	9.45	—†
Fndc5	1.11	0.22	1.08

Comparison of the expression levels of myokine genes in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†", values of all samples were too small to compare.

Overexpression of *Il15* in mice decreased their visceral fat weight, whereas *Il15*-knockout mice showed increased fat ; this suggests that *Il15* regulates adipocyte metabolism (28). Furthermore, IL-15-treated rats expressed uncoupling protein 3 (*Ucp3*) at higher levels and showed decreased lipid accumulation in adipose tissue compared to untreated controls (29). Ucp3 is a mitochondrial protein that regulates fatty acid export and/or mitochondrial proton conductance, and overexpression of Ucp3 leads to fat loss (30, 31). *Ucp3* expression levels were higher (9.47-fold) in exercised SPORTS rats than in sedentary SPORTS rats. Moreover, exercised SPORTS rats accumulate less fat compared to WT rats (7). Taken together, in SPORTS rats, physical exercise promotes *Il15* expression, which may cause less fat accumulation through Ucp3 up-regulation.

Effects of genetic mutation on gene expression

The genetic architecture for voluntary exercise is considered to be complicated and many genes seemed to be involved in this trait (6). In the case of SPORTS rats, NE accumulation in the extracellular fluid of hippocampus may influence exercise behavior (10). NE is a catecholamine and functions as a neurotransmitter. NE levels are regulated by synthesis from tyrosine and degradation via deamination by monoamine oxidases (MAO) and/or methylation by catechol-*O*-methyltransferase (COMT) (32). Morishima *et al*. demonstrated that NE levels were increased in the extracellular region of the hippocampus of SPORTS rats and that MAOA protein levels and activity were lower than those in control rats. Both the expression level and activity of COMT were similar to those of WT. Furthermore, administration of clorgyline, a specific inhibitor of MAOA, into WT rats induced a hyperactive phenotype. These findings confirmed that MAOA inactivity causes hyperactivity in SPORTS rats (9).

In our microarray analysis data, genes related to NE synthesis, such as *tyrosine hydroxylase* (*Th*, 1.44-fold) and *dopa decarboxylase* (*Ddc*, 1.98-fold), were expressed at higher levels in sedentary SPORTS rats than in WT rats (Fig. 4, Table 10). These results

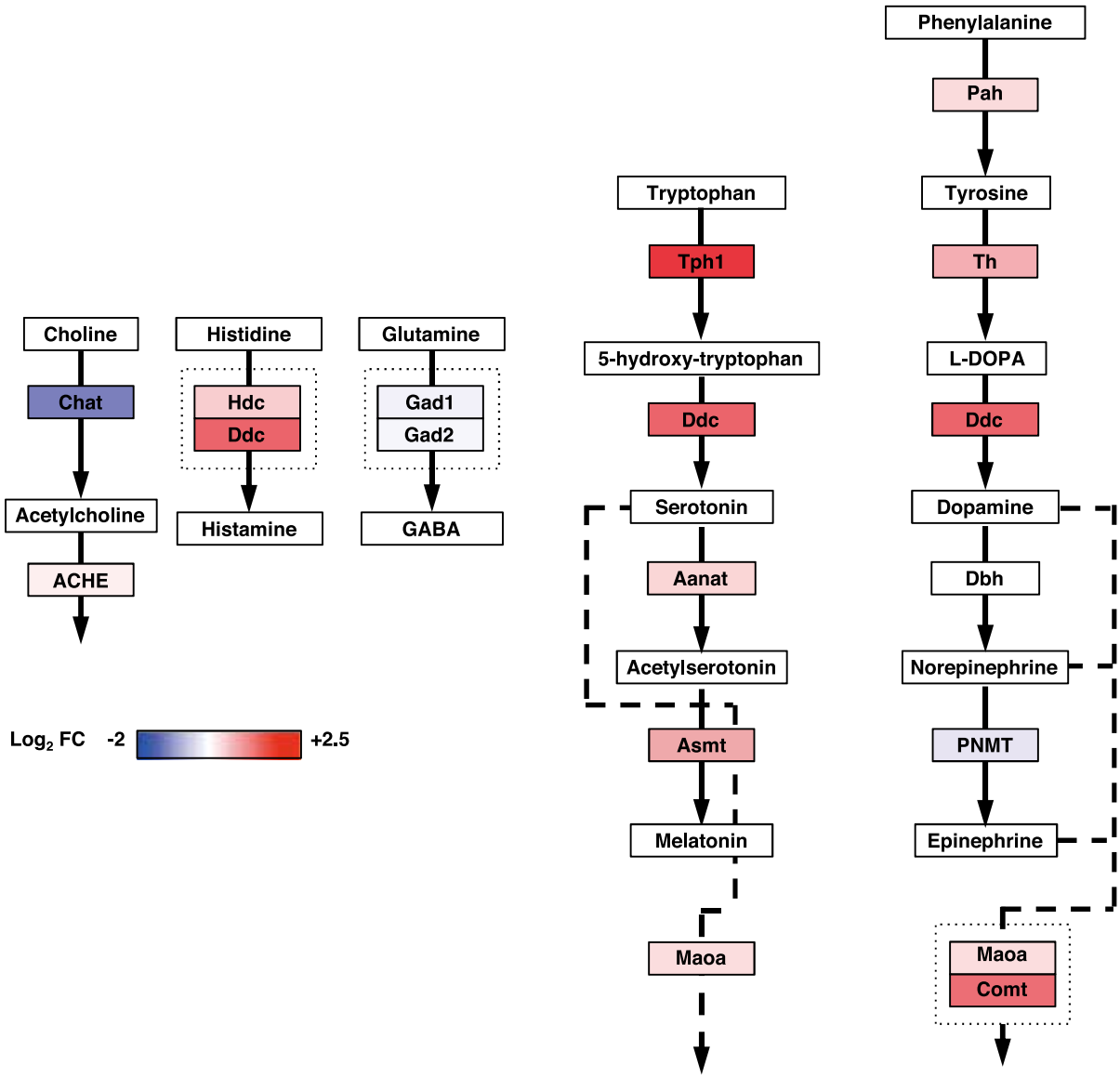


Fig 4. Catecholamine metabolic pathway
Gene expression of biogenic amine pathway in the brain tissue of sedentary SPORTS rats compared to that of WT rats and illustrated based on the biogenic amine pathway map of WikiPathway. Up-regulated genes in sedentary SPORTS rats compared to sedentary WT rats are colored in red and down-regulated genes in blue.

are consistent with previous studies that demonstrated higher expression level of NE, in extracellular region of hippocampus, although *Dopamine b-hydroxylase* (*Dbh*) expression levels showed individual variations among SPORTS rats and MAOA expression levels were not different between SPORTS rats and WT rats.

Interestingly DAVID analysis of down-regulated genes in the comparison between sedentary SPORTS rats and sedentary WT rats showed that the cluster group hormone activity was ranked the highest (Table 7B) and that *Dopamine receptor D1* (*Drd1*) and *Pmch* were included in this cluster.

Drd1 and *Nescient helix–loop–helix 2* (*Nhlh2*) are candidate factors that regulate physical activity (32). *Drd1* expression levels in the brain tissue of SPORTS rats were lower than those in WT rats, a result that was consistent with a previous report that showed higher *Drd1* gene expression in active male mice than in low active mice (Table 11) (33). This report suggests that *Drd1* may influence the regulation of exercise activity in SPORTS rats. In our microarray data, *Drd1* down-regulation was observed in the comparison between sedentary SPORTS rats and sedentary WT rats. Therefore, the difference in expression levels is not because of exercise but a genetic difference between them. However, signal intensities of *Drd1* in sedentary SPORTS rats were quite low and difficult to confirm. Molecular details remain to be determined in the next study.

Table 10. Comparison of the expression levels of biogenic amine pathway

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Th	0.98	1.33	1.44
Ddc	—§	—§	1.98
Dbh	—‡	—‡	4.89
Pnmt	0.90	0.67	0.89
Maoa	1.08	1.58	1.12
Comt	3.56	0.78	1.92
Aanat	—‡	—¶	1.16
Asmt	—‡	—‡	1.37
Gad1	—‡	—‡	0.91
Gad2	—†	—†	0.97
Hdc	0.50	0.27	1.43
Chat	—‡	—‡	1.04
Ache	1.30	5.10	1.06

Comparison of the expression levels of genes related to the biogenic amine pathway in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†," this gene did not appear in the list ; "‡," values of all samples were too small to compare ; "§," interpretation is difficult because the data varies greatly depends on the probes ; and "¶," value of Ss was too small to compare.

Table 11. Comparison of the expression levels of voluntary exercise-related genes

Gene symbol	Muscle		Brain
	Ss vs. C	Se vs. C	Ss vs. C
Pmch	—†	—†	0.42
Drd1	—†	—†	—#
Nhlh2	—‡	—§	—¶
Insig2	0.90	0.90	1.16
Socs2	0.92	1.41	0.83
Il15	0.82	12.43	0.70

Comparison of expression levels of voluntary exercise-related genes in exercised SPORTS rat, sedentary SPORTS rats, and WT rats (muscle tissue, $n = 1$; brain tissue, $n = 3$). Fold changes in each comparison are listed. Se, exercised SPORTS rat ; Ss, sedentary SPORTS rats ; C, control WT rats. "†," values of all samples were too small to compare ; "‡," value of C was too small to compare ; "§," value of Se was too small to compare ; "¶," interpretation is difficult because the data varies greatly depends on the probes ; and "#," value of Ss was too small to compare.

Zhou *et al*. reported that *Pmch* is related to voluntary exercise, showing that *Pmch*-knockout mice run long distances voluntarily (33). Moreover, *Pmch*-deficient rodents are lean, smaller, and have a low body fat mass (34). *Pmch*-deficient rats also show lower NE turnover (34). MCH, the degradation product of PMCH, is regarded as a mediator of energy homeostasis (34). Our microarray data showed *Pmch* down-regulation in the brain tissue of sedentary SPORTS rats (0.42-fold) compared to WT (Table 11) and that the physiological phenotype of SPORTS rats was similar to that of the *Pmch*-knockout mice. These results imply that *Pmch* down-regulation might affect some parts of SPORTS rat phenotypes.

We analyzed genome-wide gene-expression profiles of brain and skeletal muscle tissue of SPORTS rats compared to WT rats. We could dissect the phenotypes into two mechanisms, namely genetic changes and metabolic adaptation, while the causative gene(s) of the SPORTS rat phenotypes remained to be determined. Further studies of the phenotype and genotype correlation as well as the epigenome of SPORTS rats will help to understand the molecular basis of the beneficial effects of the exercise on our human health and to improve our quality of life.

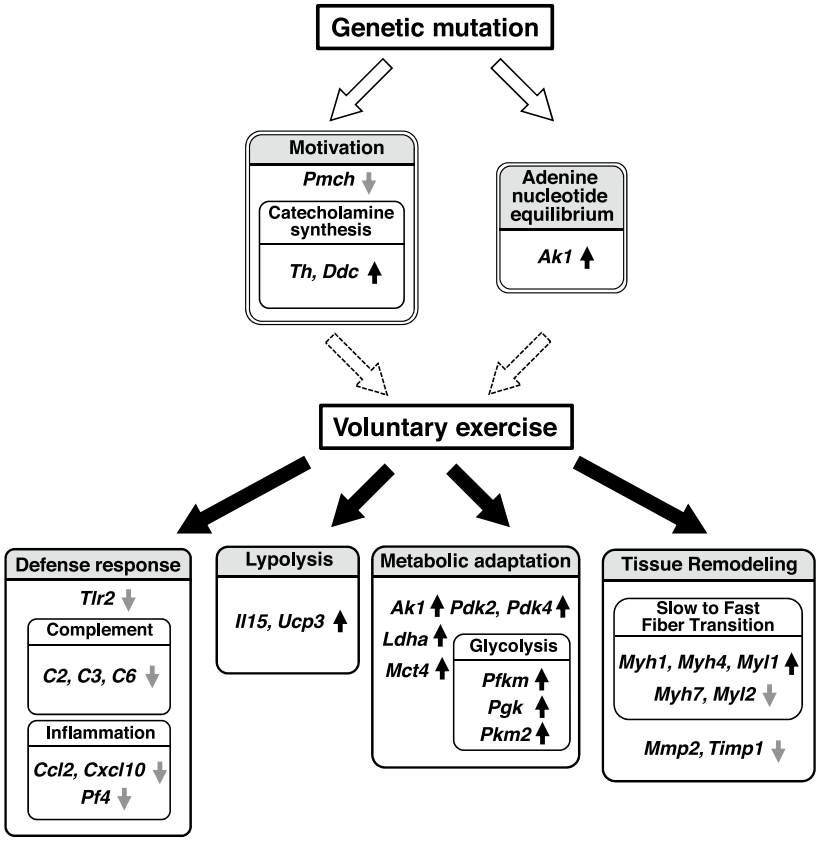


Fig 5. The effects of genetic mutation and exercise on the gene expression of SPORTS rats
Characteristic gene expressions and functional clusters in SPORTS rats are summarized. Functional clusters of brain tissue are shown in double-line squares and those of skeletal muscle in single-line squares. The effects of genetic mutation(s) and exercise are indicated by white- and black-arrows, respectively. Possible effects of motivation and adenine nucleotide equilibrium are shown as dotted white arrows. Up- and down-regulations of gene expression are depicted by upward- and downward-arrows in each box.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

Microarray analysis of skeletal muscle tissue was supported by Support Center for Advanced Medical Sciences, Institute of Biomedical Sciences, Tokushima University Graduate School.

AUTHOR CONTRIBUTIONS

T.H., Y.M., K.M., A.T., and H.H. performed the experiments ; T.H., Y.M., K.M., A.T., H.H., H.S., and T.N. analyzed data and interpreted the results of experiments ; T.H. prepared the figures ; T.N. ; T.H., H.S., and T.N. drafted the manuscript ; T.H., Y.M., K.M., A.T., H.H., H.S., and T.N. approved the final version of manuscript ; T.N. was responsible for the conception and design of the research.

FUNDS

This work was supported by Operating Support Funds from Tokushima University.

ETHICAL APPROVAL

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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