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Novel miRNAs and their Target Gene Networks from Hippocampi of Rats that Performed Regular Exercise: An *In Silico* Study

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MicroRNAs (miRNAs) have been previously described to regulate gene expression. To determine whether such non-coding RNA regulates gene activities in the brain as a consequence of regular exercise, expressed sequence tags (ESTs) derived from a related study by one of the authors on the hippocampi of Swiss Wistar rats that were trained to run on a treadmill were subjected to a computational analysis. Three putative miRNAs homologous to mmu-miR-574-5p and rno-miR-297 have been detected and their possible brain-specific target genes were predicted. Ingenuity Pathways Analysis (IPA)-generated interactomes of the miRNA target genes underscore the role of non-coding RNA in modulating neurogenesis, neuronal cell death and long-term depression. Our elucidation of some molecular networks mediated by these miRNAs offers insights to the well-established effects of physical activity on learning and memory.

Key Words: interactome, molecular networks, neuroscience, systems biology, treadmill running

INTRODUCTION

Physical exercise has been proven to be generally good for the health. It is widely accepted that regular physical activity can contribute to a reduction in the incidence of obesity (Welk & Blair 2000), cardiovascular disease, and osteoporosis (Kemper et al. 2000). It has also been shown that physical exercise has salutary effects on brain function: ameliorating age-associated cognitive losses (Hillman et al. 2006); improving synaptic plasticity, hippocampal neurogenesis, cognition response, and learning (Etnier et al. 1997; van Praag et al. 1999; Colcombe et al. 2006). These effects of running on the brain are mainly associated with changes in gene expression. Molteni et al. (2002) observed from rats that underwent voluntary exercise the up-regulation of brain-derived neurotrophic factor (BDNF) together with those involved in synaptic trafficking (e.g., synapsin I, synaptotagmin and syntaxin), signal transduction pathways (e.g., Calcium/calmodulin-dependent protein kinase II, CaM-KII; mitogen-activated/extracellular signal-regulated protein kinase, MAP-K/ERK I and II; protein kinase C, PKC-D), the glutaminargic systems (N-methyl-Daspartate receptor, NMDAR-2A and NMDAR-2B and excitatory amino acid carrier 1, EAAC1); and down-regulation of genes related to the gamma-aminobutyric (GABA) system (i.e., GABAA receptor, glutamate decarboxylase GAD65). Cavallaro et al. (2002), on the other hand, found differential expression in groups of genes related to memory and learning in mice. Among these are those involved in microtubule formation and cell signaling.

The discovery of microRNAs has opened new vistas in the understanding of gene regulation in recent years. MicroRNAs (miRNAs) are a class of non-coding RNAs

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whose products are ~22 nucleotides (nt) long. They play important roles in the regulation of translation and degradation of mRNAs. This is achieved through base pairing to partially complementary sites in the untranslated regions (UTRs) of the mRNA (Griffiths-Jones 2003).

MiRNAs have a unique feature of folding into characteristic hairpin loop structures in their precursor form. The hairpin loop consists of a head (the loop) and a pair of arms. The mature miRNA sequence is located on one arm and the sequence located directly on the other arm is called the miRNA* sequence (Preall et al. 2006). In this study, it was found through an *in silico* approach that miRNAs may play a role in regulating genes expressed in the brain after chronic exercise on a treadmill.

MATERIALS AND METHODS

Source of Microarray Data

In silico analysis was done on a microarray data set derived from 11-week-old male Swiss Wistar rats from one group (n=4-6) that underwent running for 6 weeks on a treadmill (40 m/min per session, 1 hr per session, 5 sessions per week) and from an equal number of animals on a stationary treadmill (negative control). The rats were sacrificed by decapitation three days after the final exercise activity to remove RNA signals that represent acute response to stress.

Total RNA were then extracted from the hippocampi with QIAGEN RNeasy Mini Kit (QIAGEN, MD), and analyzed spectrophotometrically and visually on agarose gel to check the yield and quality. Total RNA from 3 hippocampi (150 ng from each animal) were pooled, labeled with Cy-3 or Cy-5 using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent) and hybridized on a rat 44K whole genome oligo DNA microarray chip (G4131A, Agilent Technologies, Palo Alto, CA) following the manufacturer's procedure. After washing, the microarrays were scanned using an Agilent Microarray scanner G2565BA. A dye-swapping procedure, where labeling was performed on reverse, was done to remove dye bias.

For detection of differentially expressed genes between control and exercised samples, each slide image was processed using Agilent Feature Extraction ver.8.1.1. This software selected a probe set by rank consistency filter for dye-normalization, and normalization was performed by LOWESS (locally weighted linear regression). Then, genes showing expression change with differences at least 1.5-fold in both dye-swapped slides were selected (Hirano et al. 2008). All animal experiments, following ethical guidelines on animal care and treatment were performed at the Laboratory of Exercise Biochemistry, University of Tsukuba, Japan and microarray analysis was done by the Human Stress Group, National Institute of Industrial Science and Technology (AIST), Japan. A separate paper, which includes, among many others, data from which the ESTs were derived for this paper and which were not shown, is being prepared (Deocaris et al. in preparation).

MicroRNA Identification

Sequences of the transcriptome data set in the form of ESTs were manually obtained from the NCBI database using Entrez (NCBI 2004). The steps in homology search and secondary structure prediction used for miRNA identification in this study are shown in Figure 1. Homologous EST and known mature miRNA sequences of three species in the miRBase database were found using BLAST. Each EST sequence was aligned individually against known *Rattus norvegicus, Mus musculus*, and *Homo sapiens* miRNA and only those ESTs with a maximum of four mismatches with the query sequence were retained for further analysis. The secondary structures of the resulting mature miRNAs were then predicted using the web-based software mFold 3.2 (Zuker et al. 1999; Zuker 2003). This program is available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgibin/rnaform1.cgi.

A candidate miRNA sequence was considered if it met the following criteria: (1) the predicted mature miRNA had no more than four nucleotide substitutions when aligned with known *H. sapiens, M. musculus*, and *R. norvegicus* mature miRNAs; (2) the RNA sequence can fold into an appropriate stem-loop hairpin secondary structure; (3) a mature miRNA sequence site is present in one arm of the hairpin structure; (4) there is no loop or break in the miRNA* sequence; and (5) the predicted secondary structure has a negative minimum free folding energy (MFE; ΔG kcal/mol) and 30–70% Adenine+Uracil content (Zhang et al. 2006). These criteria reduced false positives and required that the predicted miRNAs fit the criteria proposed by Ambros and co-workers (2003).

ESTs were used as the basis of the miRNA search because they provide a robust sequence resource that can be exploited for gene discovery, genome annotation, and comparative genomics (Rudd 2003). The use of ESTs gave way for an indirect yet reliable analysis of gene expression in rats which underwent physical exercise. ESTs have also been utilized in similar studies by other researchers, such as Zhang et al. (2005) and Li et al. (2006), in discovering new precursor and mature miRNAs.

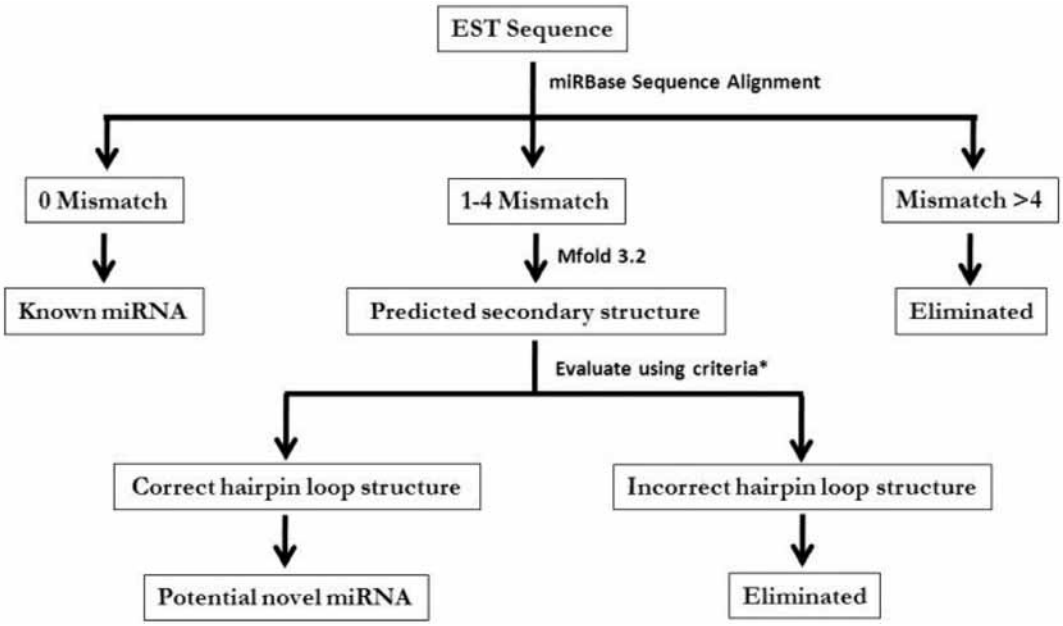

Figure 1. A schematic diagram of the over-all procedure used to identify and analyze miRNAs.

*modified from Lai et al. (2003) and Zhang et al (2006). See text for details.

In this study, an EST sequence was scanned against known miRNA sequences in the miRBase Database using BLAST. Resulting scores and E-values aided in determining which alignment results were significant. Matches with the highest scores were selected as long as the E-values did not exceed the threshold of 10.

Aside from considering rat miRNA alignment results, EST alignments with mouse miRNAs were also selected because the two organisms are very closely related, and miRNAs that are expressed in one of the organisms could be evolutionarily conserved in the other organism as well. Human miRNAs were also included as part of the search, to explore whether rat miRNAs are conserved within the human genome. This way, inferences can be made as to how the potential novel miRNAs will affect different organisms as a result of a specific physical exercise regimen.

Secondary structure prediction served to filter the candidate miRNAs from the previous step by eliminating near-positive outputs. Even if other short RNA sequences can exhibit miRNA functions, only miRNAs can form stem-loop precursors. This process also removed resulting miRNA matches from the BLAST search that were due to chance sequence similarities. The structures deemed qualified for the succeeding steps were those which fully satisfied the criteria proposed by Zhang et al. (2006) and Lai et al. (2003).

Potential miRNA Target Prediction

Using known miRNA sequences as input, the resulting putative miRNAs were tested against the miRBase Target database (miRBase Targets 2006) for brain-specific gene targets. This particular step served to provide information on potential targets of the putative miRNAs since sequence similarity with known miRNAs exists.

The MiRanda algorithm was used for target prediction. This computational method is high-throughput but has the problem of having too many false positives. Targets genes are predicted on the basis of three properties: sequence complementarity, free energy of RNA-RNA duplexes, and conservation of target sites in related genomes. Most likely, not all of the predicted targets for a miRNA represent true biological targets. In fact, only a few of these have been confirmed as either positive or negative. As accurate target prediction and validation are still major obstacles in miRNA research (Zhang et al. 2007), we undertook a systems approach in narrowing down the miRNA targets.

In principle, since miRNAs have evolved to simultaneously regulate their targets by partial complementation, it is likely that the biological effect is carried out through an *en bloc* machinery. That is, the gene targets that have the highest probabilities of being the miRNA target would likely belong to a network having a consistent biological function. Thus, a gene network analysis was carried out using the IPA knowledge database and network construction tools.

Aside from determining targets of the putative miRNA, the specific proteins that the target genes code, were identified by searching the UniProt Knowledge Base (UniProtKB) (UniProt 2002).

Gene Network (Interactome) Analysis

Pathway models were generated using IPA (Ingenuity Pathways Analysis) Ingenuity® Systems, a web-based software application that allows researchers to find interactions between genes and proteins, related networks, functions and diseases, canonical pathways, as well as drug targets and biomarkers (Ingenuity Systems 2006). Focus genes inputted by the user form the "hub" of the networks, allowing for specific interactions to be drawn. Other than meeting the user-set parameters, focus genes must also interact with at least one other gene in the Ingenuity Pathways Knowledge Base (IPKB). When these criteria are met, densely connected networks are drawn (Ingenuity Systems 2006).

The list of genes targeted by the putative miRNAs was imported into the software and the data were analyzed. Networks generated by this program are scored based on the number of Network Eligible molecules they contain, that is, the algorithm maximizes the specific connectivity of each input molecule with the other input molecules. A higher score means a lower probability of finding the observed 'Network Eligible' molecules in a network by chance. As not all 'Network Eligible' molecules may interact directly, other molecules in the IPKB are added to the network, in effect merging smaller networks into a larger one (Ingenuity Systems 2006).

RESULTS

Identified Novel Putative MicroRNAs

From the 1199 ESTs identified as changed in the hippocampus following exercise (manuscript in preparation), 3 novel pututative miRNAs from 2 ESTs were identified that could potentially be expressed in the brain as a result of treadmill running (Table 1). No previously identified miRNAs were arbitrarily represented in any of the exercise-regulated ESTs. The novel miRNAs, all of which are 23 nucleotides long, were assigned as miRNA X, Y, and Z. MiRNA X has its sequence derived from the set of up-regulated genes while the other two (Y and Z) were derived from the list of down-regulated genes. Table 1 also shows the alignment of the three novel putative miRNAs to known miRNAs after searching the miRBase database. MiRNA X is closely matched to miRNA 574-5p of the mouse while miRNA Y and Z are closely related to miRNA-297 of the rat. All three putative miRNAs meet the criteria modified from Zhang et al. (2006).

Figure 2 shows the hairpin loop structures of the potential novel miRNAs generated by mFold. The mature miRNA sequences (in red) of X and Y are both located on the 5' arm of the hairpin loop while miRNA Z has its mature sequence on the 3' arm.

Predicted miRNA Targets

Target prediction analysis using the MiRanda algorithm identified 1,878 potential target genes for the 3 novel miRNAs identified. Since miRNA Y and Z are both derived from the same EST and are closely related to the same known miRNA, they have similar targets. The gene targets were filtered using UniProtKB to identify brain-specific genes, narrowing the target gene list to 89. Of these 89 brain-specific genes, 53 are targets of miRNA X and 36 are targets of miRNA Y and Z (Table 2).

IPA-generated Gene Networks (Interactomes)

The most significant network revealed by the analysis of miRNA X target genes is shown in Fig. 3A. This gene network has an IPA score of 34 and includes 13 of the 53 putative miRNA X targets as nodes (Table 2). The 13 gene targets have functions related to development (disabled homolog 1, homeobox protein Hox-A1, neurophilin-2), molecular transport (ATP-sensitive inward rectifier potassium channel 10, glutamate receptor 2, anion exchange protein

Table 1. Alignment of novel putative miRNAs with known miRNAs, and their thermodynamic properties.

miRNA	EST source	Aligned Candidate miRNA Sequence (top) against a known miRNA Sequence (bottom). Regions of mismatch are in red.	MFE (ΔG kcal/mol)	%A+U content
X	AI179443	UGUGUGUGUGUGUGUGUGUGU (miRNA X) UGAGUGUGUGUGUGUGAGUGUGU(mmu-miR-574-5p)	-29.8	50.55
Y	BE105961	AUGUGUGUGUACAUGUAUGCAUG (miRNA Y) AUGUAUGUGUGCAUGUAUGCAUG (rno-miR-297)	-30.9	62.64
Z	BE105961	AUGUAUGUAUGUGUGUAUGUAUG (miRNA Z) AUGUAUGUGUGCAUGUAUGCAUG (rno-miR-297)	-27.6	67.03

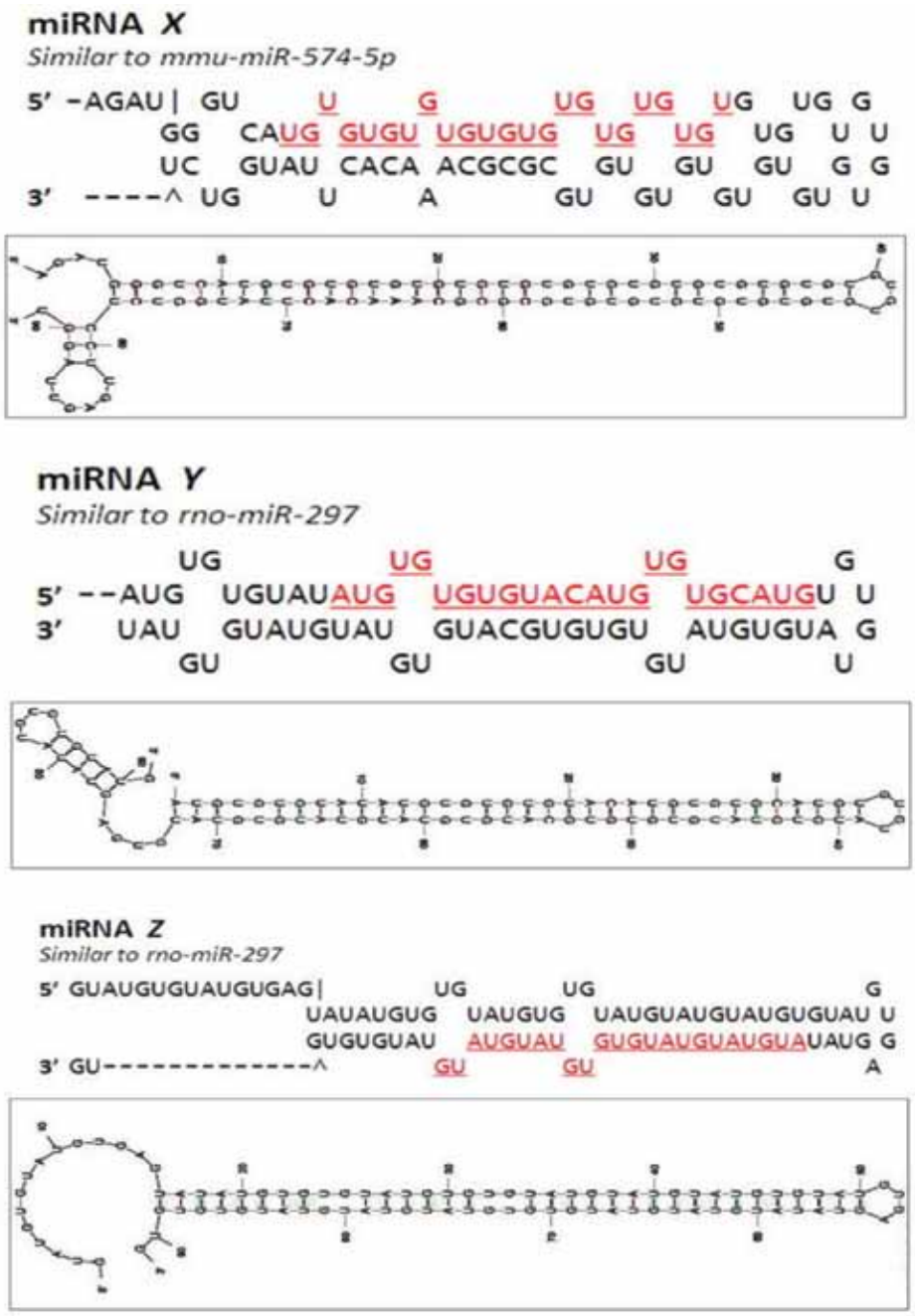

Figure 2. Hairpin secondary structures predicted by mFold 3.2 of the novel putative miRNAs with the mature miRNA sequences in red.

3), activation (GTPase-activating RapGAP domain-like 1, neurogenic locus notch homolog protein 2), receptor-mediated signaling (orexin receptor type 1, zinc finger protein 57), and protein binding (disks large homolog 4, synaptotagmin-3, tripartite motif-containing protein 3).

On the other hand, the most significant network (Fig. 3B) generated by IPA with a score of 23 contained 11 of the 36 potential target genes of miRNA Y and Z (Table 2). Similar to the previous network, the 11 targets have functions related to development (noggin), cellular growth (neurotrophin-3), molecular transport (gamma-aminobutyric acid receptor subunit beta-3, inositol 1,4,5-trisphosphate receptor type 1), catalysis

Table 2. Potential targets of the three novel putative miRNAs expressed in the hippocampus. Target genes of miRNA X are down-regulated while those of miRNA Y and Z are up-regulated.

MiRNA	Targeted Genes	Target Function	Remarks
X	Garnl1*	Activator	GTPase-activating RapGAP domain-like 1
	Notch2*	Activator, Developmental protein, Receptor	Neurogenic locus notch homolog protein 2 [Precursor]
	Pcp4	Brain-specific polypeptide	Brain-specific polypeptide PEP-19
	Clic4	Channel	Chloride intracellular channel protein 4
	Kcnh5	Channel	Potassium voltage-gated channel subfamily H member 5
	Kcnj10*	Channel	ATP-sensitive inward rectifier potassium channel 10
	Trpv1	Channel	Transient receptor potential cation channel subfamily V member 1
	Gria2*	Channel, Receptor	Glutamate receptor 2 [Precursor]
	Dab1*	Developmental protein	Disabled homolog 1
	Hoxa1*	Developmental protein	Homeobox protein Hox-A1
	Pdlim7	Developmental protein	PDZ & LIM domain protein 7
	Sncg	Developmental protein	Gamma-synuclein
	Pak3	Developmental protein, Enzyme	Serine/threonine-protein kinase PAK 3
	Ntrk1	Developmental protein, Enzyme, Receptor	High affinity nerve growth factor receptor [Precursor]
	Nrp2*	Developmental protein, Receptor, Enzyme	Neurophilin-2 [Precursor]
	B4GALNT4	Enzyme	N-acetyl-beta-glucosaminyl-glycoprotein 4-beta-N-acetylgalactosaminyl transferase 1
	Enpp5	Enzyme	Ectonucleotide pyrophos-phatase / phosphodiesterase family member 5 [Precursor]
	Hmox2	Enzyme	Heme oxygenase 2
	Pnpla7	Enzyme	Patatin-like phospholipase domain-containing protein 7
	Senp2	Enzyme	Sentrin-specific protease 2
	Tnk2	Enzyme	Activated CDC42 kinase 1
	Acvr1b	Enzyme, Receptor	Activin receptor type-1B [Precursor]
	Fgf14	Growth factor	Fibroblast growth factor 14
	Ngfb	Growth factor	Beta-nerve growth factor [Precursor]
	Cort	Hormone	Cortistatin [Precursor]
	Rln3	Hormone	Relaxin-3 [Ptrecursor]
	Nppc	Hormone, Vasoactive	C-type natriuretic peptide [Precursor]
	Phactr3	Inhibitor	Phosphatase & actin regulator 3
	Rgs9	Inhibitor	Regulator of G-protein signaling 9
	Qrfp	Neuropeptide	Orexigenic neuropeptide QRFP [Precursor]
	Atxn10	Protein binding	Ataxin-10
	Bzrap1	Protein binding	Peripheral-type benzodiazepine receptor-associated protein 1
	Crip2	Protein binding	Cysteine-rich protein 2
	Dlg4*	Protein binding	Disks large homolog 4
	Elavl4	Protein binding	ELAV-like protein 4
	Ncam1	Protein binding	Neural cell adhesion molecule 1, 140 kDa isoform [Precursor]
	Syt3*	Protein binding	Synaptotagmin-3
	Trim3*	Protein binding	Tripartite motif-containing protein 3
	Nr4a1	Receptor	Nuclear receptor subfamily 4 group A member 1
	Cry2	Receptor, Repressor	Cryptochrome-2
	Mrgprb4	Receptor, Transducer	Mas-related G-protein coupled receptor member B4
	Oprm1	Receptor, Transducer	Mu-type opioid receptor
	Adra1a	Receptor, Transducer	Alpha-1A adrenergic receptor
	Drd5	Receptor, Transducer	D(1B) dopamine receptor
	Gpr30	Receptor, Transducer	Membrane estrogen receptor
	Slc18a3	Transporter	Vesicular acetylcholine transporter
	Slc2a3	Transporter	Solute carrier family 2, facilitated glucose transporter member 3

	Slc4a3*	Transporter	Anion exchange protein 3
	Syngr1	Transporter	Synaptogyrin-1
	Iiig9	Unspecified	Uncharacterized protein C11orf66 homolog
Y and *Z*	Clic6	Channel	Chloride intracellular channel 6
	Gabrb3*	Channel	Gamma-aminobutyric acid receptor subunit beta-3 [Precursor]
	Kcnv1	Channel	Potassium voltage-gated channel subfamily V member 1
	Syt17	Channel	Synaptotagmin-17
	Itpr1*	Channel, Receptor	Inositol 1,4,5-trisphosphate receptor type 1
	Bag5	Chaperone	BAG family molecular chaperone regulator 5
	Nog*	Developmental protein	Noggin [Precursor] [Fragment]
	Acly*	Enzyme	ATP-citrate synthase
	Cacna1g	Enzyme	Voltage-dependent T-type calcium channel subunit alpha-1G
	Enpp5	Enzyme	Ectonucleotide pyrophos-phatase/phosphodiesterase family member 5 [Precursor]
	Mast1	Enzyme	Microtubule-associated serine/threonine-protein kinase 1
	Pde4b	Enzyme	cAMP-specific 3',5'-cyclic phosphodiesterase 4B
	Pla2g4a*	Enzyme	Cytosolic phospholipase A2
	Pnck	Enzyme	Calcium/calmodulin-dependent protein kinase type 1B
	Ppm1e	Enzyme	Protein phosphatase 1E
	Ppp2r2d	Enzyme	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B delta isoform
	Scp2	Enzyme	Non-specific lipid-transfer protein
	Epha6	Enzyme, Receptor	Ephrin type-A receptor 6 [Precursor]
	Epha8	Enzyme, Receptor	Ephrin type-A receptor 8
	Ntf3*	Growth factor	Neurotrophin-3 [Precursor]
	App*	Inhibitor	Amyloid beta A4 protein [Precursor]
	Znf354c*	Inhibitor, Repressor	Zinc finger protein 354C
	Fgfbp1	Protein binding	Fibroblast growth factor-binding protein 1 [Precursor]
	Kiss1*	Protein binding	Metastasis-suppressor KiSS-1 [Precursor]
	Pard3	Protein binding	Partitioning-defective 3 homolog
	Raver1	Protein binding	Ribonucleoprotein PTB-binding 1
	Sep15	Protein binding	15 kDa selenoprotein
	Sfrs12	Protein binding	Splicing factor, arginine/serine-rich 12
	Nr4a1*	Receptor	Nuclear receptor subfamily 4 group A member 1
	Cntn3	Receptor	Contactin-3
	Oprs1	Receptor	Sigma 1-type opioid receptor
	Sv2a	Receptor	Synaptic vesicle glycoprotein 2A
	Eltd1	Receptor, Transducer	EGF, latrophilin and seven transmembrane domain-containing protein 1 [Precursor]
	Oprm1*	Receptor, Transducer	Mu-type opioid receptor
	Prokr2	Receptor, Transducer	Prokineticin receptor 2
	Kalrn	Releasing factor, Enzyme	Kalirin

*node in resulting IPA network

(ATP-citrate synthase, cytosolic phospholipase A2), inhibition (amyloid beta A4 protein, zinc finger protein 354C), receptor-mediated signaling (nuclear receptor subfamily 4 group A member 1, mu-type opioid receptor) and protein binding (metastasis-suppressor KiSS-1).

DISCUSSION

Utilizing bioinformatics tools and databases, we identified three putative miRNAs expressed in the rat brain during treadmill running that target several proteins, some of which are related to brain activities. One miRNA found on the EST AI179443 is similar to mmu-miR-574-5p (miRNA X) while two of the novel putative miRNAs were found on the EST BE105961 (termed miRNA Y and Z).

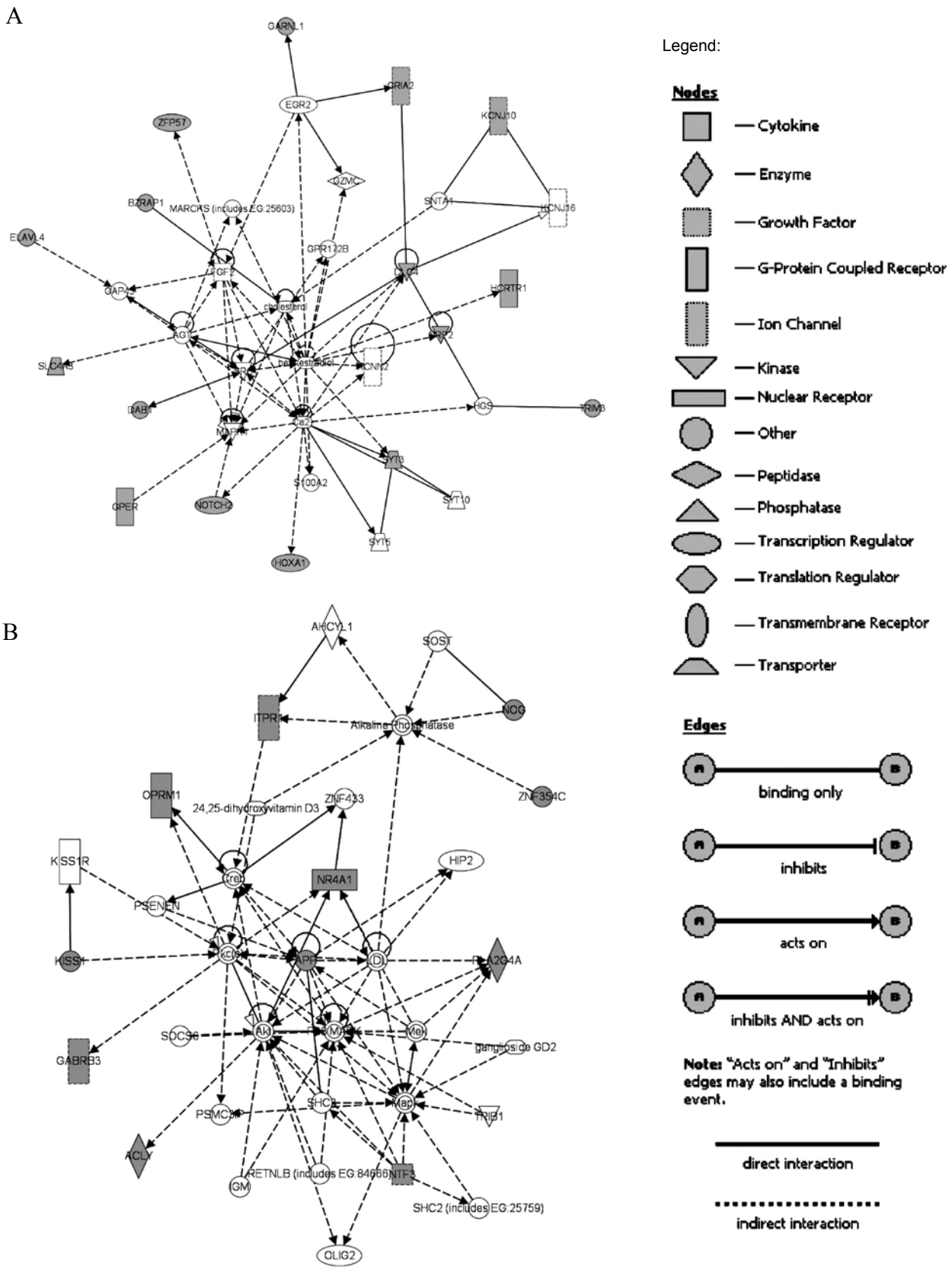

Figure 3. The most significant network of genes targeted by (**A**) miRNA X and (**B**) miRNA Y/Z that are expressed in the rat brain during treadmill running. The nodes and the nature of gene interactions are indicated in the Legend.

Table 3. The top biological functions (generated using IPA Core Analysis) of miRNA X targets.

Function	-log (p-value)	Molecules
Behavior	$6.67x10^{-7}$ - $2.2 x10^{-2}$	NGFB, OPRM1, DLG4, CRY2, QRFP, DRD5, ADRA1A, CORT, RGS9, HCRTR1, GRIA2, TRPV1, NTRK1
Nervous System Development and Function	$7.46x10^{-7}$ - $2.2 x10^{-2}$	NCAM1, NGFB, TRIM3, HMOX2, OPRM1, NRP2, NPPC, ELAVL4, DLG4, CRY2, KCNJ10, FGF14, ZFP57, DRD5, CORT, DAB1, HOXA1, HCRTR1, GRIA2, TRPV1, SYNGR1, NTRK1, PCP4
Neurological Disease	$1.27 x10^{-6}$ - $2.2 x10^{-2}$	NCAM1, NGFB, OPRM1, HMOX2, ELAVL4, DLG4, KCNJ10, FGF14, DRD5, ADRA1A, DAB1, RGS9, PAK3, ATXN10, GRIA2, TRPV1, PCP4, NTRK1
Organismal Injury and Abnormalities	$1.27 x10^{-6}$ - $2.2 x10^{-2}$	NGFB, NRP2, DRD5, OPRM1, ADRA1A, GRIA2, TRPV1
Gastrointestinal Disease	$1.02 x10^{-5}$ - $9.58 x10^{-3}$	DRD5, OPRM1, CORT, TRPV1
Inflammatory Disease	$1.02 x10^{-5}$ - $1.73 x10^{-2}$	NGFB, OPRM1, CORT, KCNJ10, TRPV1, NR4A1
Cancer	$1.34 x10^{-5}$ - $2.33 x10^{-2}$	NCAM1, NGFB, ADRA1A, HOXA1, SNCG, PAK3, NOTCH2, TNK2, NR4A1, NTRK1
Cell-To-Cell Signaling and Interaction	$1.34 x10^{-5}$ - $2.2 x10^{-2}$	NCAM1, NGFB, NRP2, DRD5, CORT, DLG4, HCRTR1, GRIA2, NTRK1
Cellular Function and Maintenance	$1.34 x10^{-5}$ - $1.47 x10^{-2}$	NCAM1, NGFB, NOTCH2, NTRK1
Skeletal and Muscular Disorders	$1.34 x10^{-5}$ - $7.4 x10^{-3}$	NGFB, NPPC, NTRK1
Cell Morphology	$4.02 x10^{-5}$ - $2.2 x10^{-2}$	NCAM1, NGFB, NRP2, SNCG, NPPC, DLG4, ELAVL4, KCNJ10, TNK2, DRD5, HOXA1, PAK3, CLIC4, GRIA2, SYNGR1, NTRK1, NR4A1
Tissue Development	$4.02 x10^{-5}$ - $2.3 x10^{-2}$	NCAM1, NGFB, NRP2, NPPC, HOXA1, DAB1, TRPV1, ACVR1B, NTRK1
Psychological Disorders	$5.05 x10^{-5}$ - $2.2 x10^{-2}$	OPRM1, DRD5, ADRA1A, ELAVL4, DLG4, PCP4
Cell Cycle	$8.02 x10^{-5}$ - $2.23 x10^{-2}$	NGFB, ADRA1A, NOTCH2, NR4A1
Endocrine System Disorders	$8.02 x10^{-5}$ - $2.2 x10^{-2}$	NCAM1, NGFB, ADRA1A, PAK3, NTRK1
Lipid Metabolism	$8.02 x10^{-5}$ - $1.84 x10^{-2}$	QRFP, NGFB, ADRA1A, NPPC, NTRK1
Small Molecule Biochemistry	$8.02 x10^{-5}$ - $1.84 x10^{-2}$	NGFB, SLC18A3, HMOX2, OPRM1, NPPC, DLG4, QRFP, DRD5, ADRA1A, RGS9, GRIA2, NTRK1, RLN3
Cellular Development	$1.16 x10^{-4}$ - $2.2 x10^{-2}$	NCAM1, NGFB, NRP2, NPPC, NOTCH2, PDLIM7, KCNJ10, TNK2, CRIP2, HOXA1, ADRA1A, PAK3, CLIC4, ACVR1B, NR4A1, NTRK1
Cell Signaling	$1.65 x10^{-4}$ - $1.95 x10^{-2}$	NCAM1, NGFB, OPRM1, NPPC, DLG4, NOTCH2, TNK2, QRFP, DRD5, CORT, ADRA1A, DAB1, RGS9, PAK3, HCRTR1, GPER, GRIA2, TRPV1, RLN3, NTRK1
Molecular Transport	$1.65 x10^{-4}$ - $1.95 x10^{-2}$	NCAM1, NGFB, SLC18A3, OPRM1, NPPC, DLG4, KCNJ10, QRFP, DRD5, ADRA1A, CORT, GRIA2, TRPV1, RLN3
Nucleic Acid Metabolism	$1.65 x10^{-4}$ - $1.84 x10^{-2}$	OPRM1, DRD5, ADRA1A, NPPC, RGS9, DLG4, RLN3
Genetic Disorder	$4.76 x10^{-4}$ - $7.4 x10^{-3}$	OPRM1, DRD5, RGS9, ATXN10
Cell Death	$1.02 x10^{-3}$ - $2.2 x10^{-2}$	NGFB, OPRM1, HMOX2, NPPC, TRPV1, NR4A1,NTRK1
Cellular Assembly and Organization	$1.19 x10^{-3}$ - $1.84 x10^{-2}$	NCAM1, NGFB, NRP2, HOXA1, DAB1, DLG4, RGS9, ELAVL4, GRIA2, SYNGR1, NTRK1
Cellular Movement	$1.19 x10^{-3}$ - $2.2 x10^{-2}$	NCAM1, NGFB, DRD5, OPRM1, NRP2, SNCG, DAB1, HOXA1, NPPC, CLIC4, GRIA2, TRPV1
Immunological Disease	$1.56 x10^{-3}$ - $2.2 x10^{-2}$	NGFB, OPRM1
Cardiovascular System Development and Function	$1.62 x10^{-3}$ - $2.2 x10^{-2}$	QRFP, DRD5, NRP2, ADRA1A, NPPC, TRPV1
Cellular Growth and Proliferation	$2.41 x10^{-3}$ - $2.27 x10^{-2}$	NCAM1, NGFB, NRP2, OPRM1, SNCG, NPPC, NOTCH2, CRIP2, CORT, HOXA1, ADRA1A, GPER, NTRK1, NR4A1

Reproductive System Disease	2.41×10^{-3} - 1.11×10^{-2}	NGFB, HOXA1, SNCG, NR4A1, NTRK1
Digestive System Development and Function	3.32×10^{-3} - 1.82×10^{-2}	QRFP, OPRM1, HCRTR1, TRPV1, RLN3
Cardiovascular Disease	3.71×10^{-3} - 1.99×10^{-2}	NGFB, OPRM1, DRD5, ADRA1A, GRIA2
Cellular Compromise	3.71×10^{-3} - 1.11×10^{-2}	NCAM1, SNCG, KCNJ10, GRIA2
Connective Tissue Development and Function	3.71×10^{-3} - 2.2×10^{-2}	NGFB, NPPC, NTRK1
Embryonic Development	3.71×10^{-3} - 2.3×10^{-2}	NCAM1, NGFB, NRP2, NPPC, HOXA1, DAB1, SLC2A3, ACVR1B
Endocrine System Development and Function	3.71×10^{-3} - 2.2×10^{-2}	NCAM1, QRFP, NGFB
Hair and Skin Development and Function	3.71×10^{-3} - 1.84×10^{-2}	NGFB, ADRA1A
Hematological System Development & Function	3.71×10^{-3} - 1.86×10^{-2}	NGFB, CORT, NOTCH2, NTRK1, NR4A1
Immune / Lymphatic System Development and Function	3.71×10^{-3} - 1.86×10^{-2}	NCAM1, NGFB, CORT, NOTCH2, NTRK1, NR4A1

Table 4. The top biological functions (generated using IPA Core Analysis) of miRNA Y and Z targets.

Function	-log(p-value)	Molecules
Lipid Metabolism	2.28×10^{-6}-1.36×10^{-2}	SCP2, KISS1, PROKR2, PLA2G4A, NTF3, ACLY, OPRS1, APP
Molecular Transport	2.28×10^{-6}-1.36×10^{-2}	SCP2, PROKR2, KISS1, OPRM1, PLA2G4A, NTF3, OPRS1, ITPR1, APP
Small Molecule Biochemistry	2.28×10^{-6}-1.36×10^{-2}	SCP2, KISS1, PROKR2, PLA2G4A, NTF3, SV2A, ACLY, OPRS1, APP
Organismal Injury and Abnormalities	1.54×10^{-5}-1.36×10^{-2}	OPRM1, PLA2G4A, GABRB3, PDE4B, NOG, OPRS1, CACNA1G, APP
Cardiovascular Disease	2.98×10^{-5}-1.36×10^{-2}	OPRM1, PLA2G4A, PDE4B, OPRS1, CACNA1G, APP
Neurological Disease	2.98×10^{-5}-1.36×10^{-2}	PARD3, OPRM1, GABRB3, NTF3, PDE4B, SV2A, OPRS1, BAG5, APP
Nervous System Development and Function	4.31×10^{-5}-1.48×10^{-2}	PARD3, EPHA8, GABRB3, KALRN, NTF3, SV2A, OPRS1, NOG, CNTN3, ITPR1, APP
Cellular Function/Maintenance	4.96×10^{-5}-1.36×10^{-2}	NTF3, ITPR1, APP
Hematological Disease	4.96×10^{-5}-1.19×10^{-2}	SCP2, OPRM1, PLA2G4A, PDE4B, OPRS1, APP, NR4A1
Renal and Urological Disease	4.96×10^{-5}-4.96×10^{-5}	OPRM1, OPRS1
Tissue Morphology	7.43×10^{-5}-1.36×10^{-2}	NTF3, NOG, APP
Psychological Disorders	1.38×10^{-4}-6.98×10^{-4}	OPRM1, GABRB3, OPRS1, APP
Genetic Disorder	1.78×10^{-4}-1.13×10^{-2}	OPRM1, OPRS1, NOG, APP
Cell Morphology	2.22×10^{-4}-1.36×10^{-2}	SCP2, PLA2G4A, KALRN, NTF3, NOG, APP
Cellular Assembly and Organization	2.22×10^{-4}-1.44×10^{-2}	SCP2, PARD3, EPHA8, KALRN, NTF3, ITPR1, APP
Cell-To-Cell Signaling and Interaction	2.71×10^{-4}-1.45×10^{-2}	EPHA8, NTF3, SV2A, CNTN3, APP
Skeletal and Muscular System Development and Function	2.71×10^{-4}-1.36×10^{-2}	PLA2G4A, GABRB3, NOG, CACNA1G, APP
Cell Death	4.46×10^{-4}-1.36×10^{-2}	OPRM1, PLA2G4A, NTF3, ITPR1, APP, NR4A1
Cellular Movement	1.25×10^{-3}-1.42×10^{-2}	KISS1, OPRM1, EPHA8, PLA2G4A, NTF3, PDE4B, NOG, APP
Behavior	1.33×10^{-3}-1.48×10^{-2}	OPRM1, GABRB3, NTF3, OPRS1, APP
Auditory and Vestibular System Development and Function	2.28×10^{-3}-6.81×10^{-3}	NTF3
Cancer	2.28×10^{-3}-1.23×10^{-2}	KISS1, PLA2G4A, KALRN, NTF3, FGFBP1, NOG, CACNA1G, APP, NR4A1
Cell Signaling	2.28×10^{-3}-1.13×10^{-2}	KISS1, PROKR2, OPRM1, NTF3, OPRS1, ITPR1, APP
Cellular Compromise	2.28×10^{-3}-1.36×10^{-2}	PPM1E, NTF3, BAG5, APP

Cellular Development	2.28×10^{-3}-1.36×10^{-2}	NTF3, NOG, APP
Cellular Growth and Proliferation	2.28×10^{-3}-1.36×10^{-2}	KISS1, PLA2G4A, NTF3, FGFBP1, NOG, CACNA1G, NR4A1
Connective Tissue Development and Function	2.28×10^{-3}-1.36×10^{-2}	NOG, APP
Connective Tissue Disorders	2.28×10^{-3}-1.36×10^{-2}	NOG, APP
Developmental Disorder	2.28×10^{-3}-1.36×10^{-2}	PLA2G4A, NOG, APP
Digestive System Development and Function	2.28×10^{-3}-2.28×10^{-2}	APP
Drug Metabolism	2.28×10^{-3}-2.28×10^{-2}	SV2A, OPRS1
Gastrointestinal Disease	2.28×10^{-3}-1.13×10^{-2}	OPRM1, PLA2G4A
Hematological System Development and Function	2.28×10^{-3}-1.41×10^{-2}	OPRM1, PLA2G4A, PDE4B, APP
Inflammatory Disease	2.28×10^{-3}-1.25×10^{-2}	OPRM1, PLA2G4A, PDE4B, APP, NR4A1
Metabolic Disease	2.28×10^{-3}-2.28×10^{-3}	SCP2
Nucleic Acid Metabolism	2.28×10^{-3}-1.13×10^{-2}	SCP2, ACLY
Vitamin and Mineral Metabolism	2.28×10^{-3}-1.13×10^{-2}	SCP2, KISS1, PROKR2, OPRM1, NTF3, ACLY, OPRS1, ITPR1, APP
Cardiovascular System Development and Function	4.55×10^{-3}-1.36×10^{-2}	PLA2G4A, NOG, APP
Cell Cycle	4.55×10^{-3}-1.36×10^{-2}	APP, NR4A1
Embryonic Development	4.55×10^{-3}-1.36×10^{-2}	NOG, APP
Gene Expression	4.55×10^{-3}-1.36×10^{-2}	PARD3, PLA2G4A, ZNF354C, NOG, APP
Immune Response	4.55×10^{-3}-1.41×10^{-2}	OPRM1, PLA2G4A, PDE4B, APP
Reproductive System Development and Function	4.55×10^{-3}-1.36×10^{-2}	KISS1, PLA2G4A
Viral Infection	4.55×10^{-3}-1.13×10^{-2}	APP
Organ Development	6.35×10^{-3}-9.07×10^{-3}	NTF3, NOG, APP
Endocrine System Development and Function	6.81×10^{-3}-6.81×10^{-3}	APP
Hair and Skin Development and Function	6.81×10^{-3}-9.07×10^{-3}	NOG
Visual System Development and Function	6.81×10^{-3}-1.36×10^{-2}	NOG
Renal and Urological System Development and Function	7.37×10^{-3}-7.37×10^{-3}	NTF3, APP
Immunological Disease	8.74×10^{-3}-1.26×10^{-2}	OPRM1, PLA2G4A, ITPR1, NR4A1
Post-Translational Modification	9.07×10^{-3}-9.07×10^{-3}	APP
Endocrine System Disorders	1.13×10^{-2}-1.13×10^{-2}	APP
Tumor Morphology	1.13×10^{-2}-1.13×10^{-2}	NR4A1
Amino Acid Metabolism	1.36×10^{-2}-1.36×10^{-2}	APP
Organismal Survival	1.41×10^{-2}-1.41×10^{-2}	GABRB3, NTF3, SV2A, NOG, ACLY, APP

Both of these are highly similar to the known miRNA rno-miR-297. These miRNAs may be validated in the future and added to the list of known rat miRNA sequences.

The targets of miRNA X, Y and Z have various neurological functions (Table 3 and 4). Hence, these molecules may play various roles, while being regulated by the putative miRNA after exercise, in carrying out the function of the hippocampus of long-term memory and spatial navigation. Exercise and miRNA may also have implications on neurological disorders like Alzheimer's disease, as the hippocampus is often the first part of the

brain to suffer cell death in affected individuals. Possible means of action of some molecules, under the influence of miRNAs, are presented in the sections that follow.

Gene Network (Interactome) Analysis

MiRNA X Target Genes

One of the top biological functions of target genes of this miRNA is nervous system function and development (Table 3). Thus, miRNA X may play a key role in neuregulin signaling, since its target genes are involved in this process. Neuregulin has been reported to induce reduction of NMDA (N-methyl-D-aspartate) receptor currents (Gu et al. 2005). Thus, decreased activity of the target gene Dlg4 due to increased miRNA X (discs, large homolog 4, Drosophila) in neuregulin signaling would result in increased NMDA receptor currents. This is consistent with increased Ca^{2+} (which passes through NMDA receptors) during physical exercise. Elevated passing of Ca^{2+} ions through the NMDA receptor, according to Maruoka et al. (2000), leads to neuronal death. However, they emphasized that the presence of PSD95 (postsynaptic density-95; not found in the network) protein linked with an NMDA receptor is essential for this particular signaling.

Another important gene in the molecular network is Gria2 (glutamate receptor 2). Gria2 has a positive effect on the brain by reducing long-term synaptic depression, regulated by low levels of hippocampal estradiol during physical exercise. Reza Zamani et al. (2000) reported that high estradiol levels induce long-term synaptic depression by lowering the frequency threshold for LTD. Thus, low levels of estradiol, and the down-regulation of the Gria2 target gene by miRNA X, help maintain a high frequency threshold for LTD. It has yet to be experimentally determined, however, if Gria2 is regulated by exercise.

MiRNA Y and Z Target Genes

The top biological functions of miRNA Y and Z targets include neurological disease and nervous system development and function (Table 4). IPA has shown that these targets interact with kinases (MAPKs) (Figure 3B) to produce a cascade of interactions among the major molecules in the network. These may have possible implications on neuron growth and differentiation in the hippocampus, as well as the progression of neurogeneretative diseases like Alzheimer's disease (AD).

Mitogen-activated protein kinases or MAPKs (includes Mapk, p38, and Mek), when activated through phosphorylation, participate in signal transduction pathways that control intracellular events including acute responses to hormones and major developmental changes in organisms (Pearson et al. 2001; Ji & Suter 2007). Such phosphorylation of Mapk in oligodendrocyte progenitors and oligodendrocytes is induced by Nerve growth factor and Neurotrophin-3 (Ntf3, a target for miRNA Y and Z) (Cohen et al. 1996). This is apparent in Figure 3B. Ntf3 has been reported to help support the survival and differentiation of existing neurons, encourage the growth and differentiation of new neurons and synapses, and play a role in the regulation of glial development in the CNS (Maisonpierre et al. 1990). The activation of Mapk by Ntf3 in oligodendrocytes might lead to the proliferation of this type of neuron. Since miRNA Y and Z were shown to be down-regulated as a result of physical exercise, their targets would then be up-regulated. Ntf3 up-regulation would mean increased neuron growth and differentiation and increased MAPK activity.

App, the gene which codes for amyloid precursor protein (APP), is another potential target gene that interacts with the different kinases and proteins within the network. APP is a membrane-spanning protein that is the source of the amyloid-beta (Aβ) peptide of neuritic plaques in Alzheimer's disease (AD). AD has been identified as a protein misfolding disease due to the accumulation of an abnormally folded product of APP or hyperphosphorylation of tau (τ) proteins in the brain (Hashimoto et al. 2003). It is in the hippocampus of affected individuals where damage to the brain is first seen.

LeBlanc et al. (1998) have shown that APP processing can be controlled by Protein kinase c (Pkc) through the non-amyloidogenic pathway to produce the neuroprotective sAPPα. This APP-Pkc connection is evident in the molecular network in Figure 3B. Relating this to exercise, it has been shown that physical exercise contributes to increased Pkc activity (Heled et al. 2003). Therefore, as a consequence of physical exercise, putative novel miRNA Y and Z would be down-regulated, resulting in the possible up-regulation of the App gene. Consequently, Pkc activity is also increased during physical exercise, favoring the accumulation of sAPPα, the neurotrophic and neuroprotective product.

That exercise can be effective in ameliorating the effects of AD is not new, for example, a study by Adlard et al. (2005) showed that physical exercise can function as a simple behavioral intervention sufficient to inhibit the normal progression of AD. They used a genetically modified strain of mice (TgCRND8) exhibiting AD-like pathology and examined the interaction of physical exercise with the AD cascade. Long-term physical exercise showed enhanced learning of TgCRND8 animals in a maze, with significant reductions in escape latencies over the first three (of six) trial days.

However, Greenberg and colleagues (1994) showed that the sAPPα fragment of APP can potently stimulate Mapk to phosphorylate τ in the brain. Many kinases can phosphorylate τ in vitro, but MAPKs are among the likeliest to do so in vivo. Persistent activation of Mapk by sAPPα (through mutations in the App gene) could increase phosphorylation of τ as well as other cellular elements, ultimately leading to the loss of cytoskeletal integrity and synaptic death, which are characteristics of AD. These studies suggest that the up-regulation of the App gene (through down-regulation of miRNA Y and Z because of physical exercise) to produce sAPPα would then present seemingly problematic results presenting us the limitation of bioinformatic analysis.

The IPA network functions only to display which of the genes possess established interactions with one another and it does not report which of these interactions are dominant. Considering that, we cannot verify whether the physical exercise regimen of the rats was indeed sufficient to induce increased Pkc activity resulting in production of sAPPα or to produce enough sAPPα to stimulate Mapks to phosphorylate τ. Further experiments with the sAPPα product of App and its role on the pathways of Mapk and Pkc would provide more information on how this secretory product works as a neuroprotective agent and how it can also contribute to the increased phosphorylation of τ, which is one of the landmarks of AD.

Final Remarks

Computational approaches in determining expressed microRNAs from the rat hippocampi during physical exercise can open doors for discovering other mechanisms of how physical exercise improves brain function. The IPA generated networks show us possible key points for intervention of the potential novel miRNAs, provided that they are expressed as a result of physical exercise. Moreover, since most up-regulated target genes are involved in neuron growth and differentiation, the possibility that physical exercise can be involved in enhancing memory and learning can be explored.

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