High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice

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Although exercise is critical for health, many lack the motivation to exercise, and it is unclear how motivation might be increased. To uncover the molecular underpinnings of increased motivation for exercise, we analyzed the transcriptome of the striatum in four mouse lines selectively bred for high voluntary wheel running and four non-selected control lines. The striatum was dissected and RNA was extracted and sequenced from four individuals of each line. We found multiple genes and gene systems with strong relationships to both selection and running history over the previous 6 days. Among these genes were Htr1b, a serotonin receptor subunit and Slc38a2, a marker for both glutamatergic and γ-aminobutyric acid (GABA)-ergic signaling. System analysis of the raw results found enrichment of transcriptional regulation and kinase genes. Further, we identified a splice variant affecting the Wnt-related Golgi signaling gene Tmed5. Using coexpression network analysis, we found a cluster of interrelated coexpression modules with relationships to running behavior. From these modules, we built a network correlated with running that predicts a mechanistic relationship between transcriptional regulation by nucleosome structure and Htr1b expression. The Library of Integrated Network-Based Cellular Signatures identified the protein kinase C δ inhibitor, rottlerin, the tyrosine kinase inhibitor, Linifanib and the delta-opioid receptor antagonist 7-benzylidenenaltrexone as potential compounds for increasing the motivation to run. Taken together, our findings support a neurobiological framework of exercise motivation where chromatin state leads to differences in dopamine signaling through modulation of both the primary neurotransmitters glutamate and GABA, and by neuromodulators such as serotonin.

Keywords: Exercise, gene expression, motivation, natural reward circuit, RNA-seq, selective breeding, striatum, voluntary wheel running

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Although a broad literature has established the critical importance of aerobic exercise for maintaining physical and mental health throughout the life span, average daily levels of physical activity continue to decline in western society (Brownson et al. 2005; Colcombe & Kramer 2003). If we all exercised on a regular basis – e.g. raising the heart rate and using large muscle groups for 40 min a day three times a week – we would substantially reduce the incidence of some of the most common diseases afflicting western society including obesity, heart disease, cognitive decline with aging and neurodegenerative disease (Hillman et al. 2008). A major obstacle preventing the global therapeutic application of exercise is that, for many people, the desire to exercise is low and appears to be declining (Brownson et al. 2005; Dishman et al. 1985). Moreover, it is not clear how to increase motivation for exercise. Although physical exercise can be rewarding and addictive in certain individuals, including some rodent models (Kolb et al. 2013a), suggesting that motivation for exercise can be increased (Aidman & Woollard 2003; Brene et al. 2007; Landolfi 2013; Rhodes et al. 2005), relatively few studies have investigated the neurobiological causes of increased motivation for physical activity (as reviewed in Garland et al. 2011). Thus, our understanding of how to neurobiologically increase desire to be physically active is rudimentary at best. Mouse lines selectively bred for increased voluntary wheel-running behavior provide a useful resource for discovering the neurological basis of high motivation for exercise. In the early 1990s, members of our group undertook a long-term selective breeding experiment for increased voluntary wheel-running behavior in laboratory mice to study the evolution of exercise physiological traits that support high activity levels (Swallow et al. 1998a). At the time, it was not apparent that breeding mice for increased voluntary wheel-running behavior would necessarily increase motivation for exercise. However, in retrospect, it is clear that exercise physiology and morphology – lung capacity, heart size, muscle mass and mitochondria – were not the limiting factors in initial generations determining how much individual mice ran. Instead, it was their internal motivation to want to run for longer durations and at higher speeds (Rhodes et al. 2005). Only in later generations did exercise physiological
traits begin to change (e.g. increased aerobic capacity and decreased muscle size; Garland et al. 2002; Rezende et al. 2005, but see Swallow et al. 1998b). Subsequent studies have confirmed that the mice from the selected lines display increased motivation to run as measured in experiments that manipulated the difficulty of running on wheels using obstacles (McAleer et al. 2000) or operant responses (Belke & Garland 2007).

That motivation for wheel running has increased in replicate lines of mice selectively bred for high levels of running means that frequencies of alleles for certain genes have been altered by selection to affect the development and function of neural circuits causing the increased motivation. A series of behavioral pharmacological studies suggested that dopamine D1-like signaling was altered in the mice bred for running, but which of the many molecules involved in dopamine signaling (e.g. receptors, transporters, intracellular signaling molecules, DARPP32, kinases and phosphatases) were altered from selection and how they have been altered has yet to be established. In generations 23–29, we found that the high-runner mice display opposing behavioral responses to drugs that block the dopamine reuptake transporter protein including cocaine, methylphenidate (Ritalin) and GBR 12909 (a highly specific dopamine reuptake inhibitor) (Rhodes & Garland 2003; Rhodes et al. 2001). Although these drugs tended to reduce running in mice from the high-runner lines, they either had no effect or increased running in control lines. Subsequent pharmacological studies indicated that the high-runner mice were significantly more sensitive to the effects of dopamine D1-like antagonists in reducing running, but similarly sensitive to D2-like antagonists (Rhodes & Garland 2003). We observed no differences in sensitivity to opioid receptor antagonists or selective serotonin reuptake inhibitors (e.g. fluoxetine; Li et al. 2004; Rhodes et al. 2001). These behavioral pharmacological results led us to the hypothesis that selection for increased voluntary wheel running altered dopamine signaling (Rhodes et al. 2005). However, the specific molecular, neuroanatomical and neurophysiological changes leading to the altered dopamine pharmacology remained a mystery.

In generation 29, we identified the striatum as a brain region that likely had undergone molecular changes related to increased motivation for exercise in the high-runner lines. The region of the brain with the greatest density of dopamine receptors, the striatum, was differentially activated in high-runner mice than controls, and the difference appeared related to internal and intrinsic motivation to run rather than being a consequence of the locomotor behavior (Rhodes et al. 2003). This was established in an experiment where mice were allowed 6 days of running but then on day 7 were prevented access to wheels (blocked treatment). Not only did high-runner mice display more c-Fos+ cells than controls in the striatum under the blocked condition, but also the number of cells was strongly positively correlated with distance run on the previous days, a measure of how much the mice would have run if they could have. Moreover, no differences were observed between high-runner and control lines when mice were free to run, and c-Fos+ numbers were not correlated with acute levels of running in freely running mice. The cFos is an immediate early gene transcription factor; it binds to DNA and alters the expression of many genes. The presence of high levels of cFos in neurons indicates the cell is undergoing a cascade of gene expression changes that has been referred to as the genomic action potential (Clayton 2000). Taken together, these data suggest that genomic changes occur in the striatum during the blocked condition that reflects the motivation to run. However, the specific molecular changes in the striatum, downstream of c-Fos, that orchestrate the emotional state of increased desire to run remain unknown.

Follow-up studies found no differences in concentrations of dopamine D1 or D2 receptors, or dopamine transporters, in the striatum of High-Running mice as compared with controls (unpublished data). However, the High-Running mice have larger total midbrain volumes (Kolb et al. 2013b), a particularly interesting finding given that the ventral midbrain projects dopaminergic afferents to the striatum. In generation 35, we found that High-Running mice from one of the four lines, as compared with one control line, display lower concentrations of dopamine in the dorsal raphe nucleus, the principle region where serotonergic neurons reside that project widely throughout the brain including prominent projections to the striatum (Waters et al. 2013). The high-runner mice also had lower concentrations of the dopamine metabolite DOPAC in the substantia nigra, the portion of the ventral midbrain that projects to the dorsal striatum. Further, high-runner mice had lower levels of serotonin and the metabolite 5H1AA in the substantia nigra (Waters et al. 2013). In generation 65, 5HT1a receptor agonists and antagonists differentially affected treadmill endurance and wheel-running behavior in the high-runner vs. control lines (Claghorn et al. 2016). Taken together, these data strongly suggest that genetic modifications have taken place that affect the ventral midbrain and striatum, and involve the intersection of dopamine and serotonin signaling systems. However, dopamine and serotonin signaling are exceedingly complex. Each of these signaling systems involves hundreds of different molecules involved in the coordination of the molecular cascade of events inside the postsynaptic neurons receiving the signals. Although these results strongly suggest that dopamine and serotonin signaling systems in the striatum have evolved in mice selectively bred for increased voluntary wheel-running behavior, the precise molecules affected and how they have changed at a genetic level remain unclear.

The goal of this study was to use next-generation sequencing technology to identify genes and non-coding RNAs differentially expressed in the striatum and potentially responsible for the increased motivation to run in the high-runner lines of mice. RNA-Seq was used to count RNA fragments in mice from all eight lines under the blocked running condition (see above). Modules of correlated networks of genes and non-coding RNAs were constructed and analyzed based on a number of different criteria to identify putative genes and RNAs consistent with a causal role in the motivation to run. By cross-referencing the data from this experiment with exome sequence data collected in one of the selected lines and in the founder population, we aimed to establish a potential mechanism for how a previously
established variant influences motivation to run by regulating the expression of a network of genes in the striatum. Finally, we cross-referenced our data with the Library of Integrated Network-Based Cellular Signatures (LINCS; linccloud.org; Vempati et al. 2014) database to identify putative compounds that could be used to pharmacologically manipulate levels of voluntary exercise. Identifying pharmacological agents that increase motivation for exercise could have important clinical significance for treating a range of neurological disorders as well as enhancing wellness of the general population.

Materials and methods

Experimental subjects

A total of 31 young adult male mice from generation 66 of a replicated selective breeding experiment for high voluntary wheel-running behavior were used (n=4 per line except control line 1 where n=3 because treatment was accidentally missassigned; each individual was from a different family). As previously described (Careau et al. 2013; Swallow et al. 1998a), the original progenitors were outbred, genetically variable laboratory house mice (Mus domesticus) of the Hsd:ICR strain. After two generations of random mating, mice were randomly paired and assigned to eight closed lines (10 pairs in each). In each subsequent generation, when the offspring of these pairs were ~6–8 weeks old, they were housed individually with access to a running wheel for 6 days (except during generations 22–35 when selection was relaxed as the colony was moved from Wisconsin to California; see Kolb et al. 2010). In the four high-runner lines, the highest running male and female from each family were selected as breeders to propagate the lines to the next generation. The selection criterion was total number of revolutions run on days 5 and 6 of the 6-day test. In the four control lines, a male and a female were randomly chosen from each family. Within all lines, the chosen breeders were randomly paired, except that sibling mating was avoided.

The 31 adult male mice from generation 66 used for this experiment were weaned in groups of two to four when they were 21 days old, and individually marked, following the usual procedure (Swallow et al. 1998a). When they were 35 days old (~±5 days), they were placed individually with access to wheels. Wheel running was monitored continuously for 6 days following the routine phenotyping procedure. Rooms were kept on a shifted light–dark cycle with lights on at 0000 h and off at 1200 h. On day 7 at approximately 0000 h, mice were blocked from entering the running wheel by placing a barrier to cover the tunnel connecting the wheel to the cage (Rhodes et al. 2003). Starting at 1300 h and ending at 1700 h (~1–4 h after the lights were shut off) when mice are normally most active on running wheels (Girard et al. 2001; Malisch et al. 2007), mice were removed from their cages and immediately decapitated.

Striatum dissection

All surfaces and instruments were sprayed with RNase Away (Thermo Fisher, Waltham, MA, USA). The brains were immediately placed on an aluminum platform on wet ice with ventral surface exposed. Using the olfactory tubercles and optic chiasm as landmarks, a razor blade was used to cut a coronal section ~1.7 mm thick containing the striatum. The section was then flipped horizontally and the entire striatum (dorsal and ventral) was carefully dissected from the cortex, medial septum and olfactory tubercles. The striatum was then immediately placed in a centrifuge tube on dry ice and subsequently stored at ~80°C.

RNA extraction

Tissue was homogenized with an RNase-free disposable pellet pestle (Fisher Scientific, Pittsburgh, PA, USA) and RNA was extracted using the commercially available RNaseasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). Purification of the isolated RNA included treatment with DNase I (Qiagen), according to the manufacturer's instructions. For assessing total RNA yield, aliquot samples were measured with a Qubit® 2.0 (Life Technologies, Carlsbad, CA, USA). Quality and integrity of isolated RNA samples were determined by 28S/18S RNA analysis using RNA Integrity Number (RIN) using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). The RNAs for all samples sequenced ranged from 8.5 to 9.4, above the commonly used threshold of 8.

RNA sequencing

RNA-Seq libraries were prepared using the TruSeq Stranded RNA Sample Prep Kit (Illumina, San Diego, CA, USA) with an average fragment length of 240 bp. Libraries were quantified using quantitative polymerase chain reaction, pooled in pools of 16, and multiplexed across a total of eight lanes. The libraries were sequenced on an Illumina HiSeq 2000 (Illumina) for 101 cycles in 100 bp paired-end format using a TruSeq SBS Sequencing Reagent Kit, v. 3 (Illumina). Average library sequencing depth was 58 225 473 reads and ranged from 43 248 769 reads to 70 456 861 reads. FASTQ files were generated from the raw sequencing runs using CASAVA v. 1.8.2 (Illumina). Raw sequence and processed data from this project have been uploaded to the NCBI’s GEO and are accessible at accession number GSE86076.

Bioinformatics and sequencing analysis

Reads in FASTQ format were aligned to the Mus musculus Ensembl GRCh38 genome with Ensembl (GRCm38.75 annotation using TopHat2 v. 2.0.10 (Trapnell et al. 2012). As most downstream applications use a count-based method (Anders et al. 2013), we counted reads in each sample using htseq-count from the HTSeq Python framework, v. 0.6.1 (Anders et al. 2015).

Statistics

All statistical analyses were performed in R v. 3.1.1. As in previous studies of these lines, total distance traveled on days 5 and 6 was analyzed using a mixed effects model with restricted maximum likelihood estimation, including line-type (high-runner vs. control) as a fixed effect and line (1–8) nested within line-type (n=4 lines per line-type) as a random effect. The purpose of the mixed model is to account for dependence among individuals by line and different changes resulting from selection as opposed to genetic drift (see Rhodes & Kawecki 2009). The consistency in levels of running displayed by individuals across the 6 days was assessed comparing variation in running among vs. within individuals. A log-likelihood test was conducted to compare the intercept model to one that includes the intercept plus a random effect of individuals nested within day. For all the above analyses, a P-value <5% was considered statistically significant. Statistical analysis of differential gene expression was performed on TMM-normalized values for all genes with read counts ≥1 counts per million (CPM) in at least three samples. We built two statisti- cal models using the edgeR Bioconductor package, v. 3.4.8 (Robinson et al. 2010), which assumes that residual expression values display a negative binomial distribution. Model 1 compared control with high-runner, whereas model 2 regressed gene expression on number of revolutions over the 48-h period prior to blocking wheel access. Both models used the robust implementation of tagwise disper- sion. Diagnostic plots including the first to principal components of the expression data ≥1 CPM in at least three samples, Relative Log Expression boxplots for raw and Trimmed Mean of M-values (TMM)-normalized CPM expression values and P-value distributions are displayed in Fig. S1 (Supporting information). We also analyzed the data using binary log-transformed Read per Kilobase of Transcript per Million mapped reads (RPKM) data assuming that residuals were normally distributed using a simple t-test to compare high-runner and control mice. We used a linear mixed effects model that included lines (1–8) nested within line-type (high-runner and control) entered as a random effect to account for genetic drift. Skewness, kurtosis, F statistics and P-values, and false discovery rates (FDRs) are included for these analyses in Table S1. For each of these analyses, an FDR was calculated using the Benjamini and Hochberg Method (Benjamini & Hochberg 1995) and an FDR of <5% or 10%, depending on the analysis, was considered statistically significant.

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DAVID enrichment analysis
To enrich our differential expression results comparing control and selected lines for biological systems, we used the bioinformatic annotation tool DAVID v. 6.8 (Dennis et al. 2003). This analysis used all genes at FDR < 0.10 in three sets: upregulated genes, downregulated genes or both genes upregulated and downregulated in the same gene set. We used all genes that passed the filtering threshold of ≥ 1 CPM in at least three samples as a background. DAVID clustering with signed correlation coefficients identified relevant modules and a medium stringency. We used an annotation cluster enrichment score cut-off of >1.3, which corresponds to a P-value < 0.05, as our criterion for significant enrichment.

Differential exon usage analysis
We assessed differential exon usage (DEU) using the model-based approach to read counts implemented in DEXSeq (Anders et al. 2012). Briefly, we flattened an annotation file using the flattening script included in the DEXSeq package and then used the resultant flattened annotation file to count reads in mutually exclusive exon bins. We then fitted a generalized linear model with a negative binomial distribution looking at the interaction between exon bin level expression values and selection. These were not mixed effects models, i.e. did not include line nested within line-type as a random effect. For each gene, significant interactions between selection and exon usage level indicated DEU.

Weighted gene coexpression network analysis
We performed weighted gene coexpression network analysis (WGCNA) (Langfelder & Horvath 2008). Prior to coexpression analysis, we log2-transformed data and filtered all genes displaying zero variance. Transformation was performed using voom+limma in limma v. 3.20.9. We then performed WGCNA on the transformed dataset using Pearson correlation coefficients with a soft threshold parameter for the cutreeDynamic function set to 2 and the mergeCutHeight parameter for the mergeCloseModules function set to 0.15. The resultant WGCNA yielded 60 modules with a maximum module size of 2374 genes and a minimum module size of 87 genes. WGCNA was unable to cluster 3729 genes, which were clustered into module 0. The modules from this analysis were robust: all modules showed much higher average adjacency than equal sized null modules generated from 10,000 randomizations on the gene set, and all modules displayed much higher average intramodular than extramodular connectivity after scaling these measurements for module size (Fig. S2, Table S2). The WGCNA used WGCNA v. 1.41-1 in R v. 3.1.1.

Most eigeneigenvalues were compared between control and selected mice using simple t-tests (not using mixed models with line nested within line-type entered as a random effect) and by regressing against running distance before wheels were blocked. Additionally, we compared correlation coefficients between module memberships and individual gene correlations with running within each module. A higher absolute correlation between module membership and gene-wise correlations with running implies that a module and its genes are good predictors of running.

We seeded a network with pairwise correlations between all genes in any of the four WGCNA modules whose eigengenes are significantly associated with running behavior (FDR < 0.01), modules 4, 6, 11 and 24. After setting a stringent threshold of an absolute correlation of 0.85 or better, we plotted a network of these edges using Cytoscape v. 3.2.0. The resultant network contained one large connected component, which we isolated. We plotted both the directionality of correlations and the magnitude of each edge’s correlation with behavior in the final network diagram.

LINC5 connectivity mapping
To identify pharmaceutical compounds with the novel potential to enhance motivation for voluntary aerobic exercise, we used an unbiased, data-driven approach by screening the LINC5 database against the RNA expression pattern induced by selection for voluntary exercise. The LINC5 database has been used successfully as a pattern-matching tool in drug discovery (Kunkel et al. 2011; Lamb et al. 2006; Liu et al. 2015; Qu & Raipal 2012; Wei et al. 2006). Therefore, we entered the list of differentially expressed genes at FDR P-values < 0.05, and queried the database for drugs with the highest positive connectivity scores (CSs). Connectivity ranges from −1 to +1 to reflect the default consistency, negative or positive between the RNA expression profiles. A positive CS is presumed to denote that the expression profile obtained from systematic perturbation of human cell lines with small-molecule and bioactive agents is concordant with the expression profile that underlies selection for high wheel running. Therefore, drugs with strong positive CSs are predicted to mimic the condition of high motivation for exercise. The LINCS reference gene expression database currently consists of more than 6 million expression signatures from ~5000 drug perturbations across a diverse panel of human cell lines in culture that are derived from several tissues including brain.

Results
Wheel running
Among the 31 males used in this study, mice from the high-runner lines ran significantly more than control lines on days 5 and 6 (F1,8 = 55.86, P = 2.96 × 10−4). With the exception of a single animal from selected line 7 that ran very little, no overlap in wheel-running behavior occurred between high-runner vs. control (Fig. 1). Individuals displayed consistent levels of running across the 6 days, as indicated by significant among-individual vs. within-individual variation (P = 0.0006).

Expression
A total of 522 genes were differentially expressed between high-runner and control lines at FDR < 0.05, with 287 genes upregulated in selected lines and 235 genes downregulated. The top differentially expressed gene, 5031434O11Rik (Fig. 2a), displayed no overlap between high-runner vs. control mice. The 5031434O11Rik is a long non-coding RNA (lncRNA), an antisense transcript that physically overlaps with the 5′ end of the gene Seta7 on the genome, an interaction previously observed to indicate transcriptional regulation of a target gene by an IncRNA (Katayama et al. 2005). As Seta7 is a lysine-histone-methyltransferase, the strong downregulation of 5031434O11Rik in selected animals suggests a relationship between chromatin state and running histone lysine methylation.

The strong downregulation of 5031434O11Rik in selected animals supports a relationship between chromatin state and running through histone lysine methylation. Additional highly differentially expressed genes included Gak (Fig. 2b), a gene coding a cyclin-dependent kinase whose human homologue is associated with Parkinson’s disease (Rhodes et al. 2011; Tseng et al. 2013), and Mfsd7a (Fig. 2c), a gene encoding a major facilitator superdomain, a membrane transport protein that is expressed in endothelial cells that comprise the blood–brain barrier. Full differential expression results are included in Table S1.

To enrich our expression results for ontology, we used DAVID to find annotation clusters showing significant associations with differentially expressed genes. We identified
seven annotation clusters associated with selection (Table 1). Three of these enrichment clusters were related to transcriptional regulation, two were related to cell cycle and cyclin-dependent kinases, and two related to chaperones and protein folding. These results suggest that a key signature of selective breeding is transcriptional regulation. Full DAVID enrichment results are included in Table S3.

When we tested DEU between control and high-runner lines, we identified 467 DEU events at FDR < 0.05. Although most of the DEU events we found alter the untranslated regions of their respective transcripts, we identified one particularly interesting set of DEU events that alter the translation of Tmed5, a gene coding for a protein involved in retrograde Golgi signaling. In the high-runner lines, the canonical transcript (Tmed5-001) is decreased relative to a pair of splice variants lacking the transmembrane and cytoplasmic domains (Tmed5-003 and Tmed5-004). A summary of the results is included in Fig. 3. We note that the genomic context of Tmed5, mouse cytoband 5F, was identified as a genome region of interest in previous expression work on a related strain (Saul et al. 2012).

We also found evidence of DEU in Setd7 (FDR = 2.59 × 10^{-10}), the putative target of the IncRNA 5031434O11Rik identified as the top differentially expressed gene (DEG) in the expression results. We believe the correlation between the observed DEU in Setd7 and the differential expression of 5031434O11Rik suggests that 5031434O11Rik acts through modulating Setd7 splicing; however, as there is only one transcript variant of Setd7 in the current Ensembl annotation, we cannot correlate the DEU to a functional change at this time. All DEU results reaching FDR < 0.05 are included in Table S4.

In addition to comparisons between control and high-runner lines, we found many genes whose expression was strongly correlated with distance traveled on the wheel before wheels were blocked, as this served as a
useful measure of motivation for running in a previous study (Rhodes et al. 2003). We found 922 genes whose expression was significantly correlated with distance traveled at FDR < 0.05. Interesting gene candidates included Gpr3 (Fig. 4a), a gene coding for orphaned G-protein-coupled receptor (GPCR); Slc38a2 (Fig. 4b), a gene encoding a neuronal amino acid transporter common to both glutamatergic and γ-aminobutyric acid (GABA)-ergic signaling pathways; and Htr1b (Fig. 4c), a gene encoding a metabotropic serotonin receptor implicated in multiple human disorders involving inhibition of behaviors including obsessive-compulsive disorder, Parkinson’s disease, and Tourette’s syndrome (Mas et al. 2014; Varrone et al. 2014; Yi et al. in press). These genes

**Table 1:** Significant (enrichment score > 1.3) DAVID enrichment clusters of genes at FDR < 0.1 with summary terms. Annotation clusters related generally to transcriptional regulation appear multiple times on this list.

<table>
<thead>
<tr>
<th>Annotation cluster summary term</th>
<th>Direction</th>
<th>Enrichment score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chaperone and protein folding</td>
<td>UP</td>
<td>1.72</td>
</tr>
<tr>
<td>Transcriptional repression</td>
<td>UP</td>
<td>1.64</td>
</tr>
<tr>
<td>Cyclin and cyclin-dependent kinases</td>
<td>UP</td>
<td>1.62</td>
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<tr>
<td>Transcription factors and transcriptional regulation</td>
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<td>Chaperone and protein folding</td>
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<td>1.37</td>
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<tr>
<td>Zinc finger proteins</td>
<td>UP</td>
<td>1.34</td>
</tr>
</tbody>
</table>

**Figure 3:** Differential exon usage identified in the gene **Tmed5.** (a) DEU plot comparing control and high-runner animals for the gene **Tmed5.** (b) Splice variation supported by DEU results. The canonical variant (**Tmed5-001**) is downregulated in selected animals relative to two splice variants (**Tmed5-003** and **Tmed5-004**) lacking the transmembrane and cytoplasmic domains of the canonical variant.

**Figure 4:** Gene expression regressed on behavior for key genes. Bivariate plots show expression predicted by running of (a) **Gpr3**, an orphaned GPCR, (b) **Slc38a2**, a neuronal amino acid transporter responsible for the reuptake of metabolites of both glutamate and GABA and (c) **Htr1b**, a metabotropic serotonin receptor correlated with mental health disorders involving motivated behavior.
agree with previous results suggesting high-runner lines display altered serotonin signaling in the striatum (Waters et al. 2013).

**Coexpression network analysis**
We used WGCNA, a systems biology tool designed for the *de novo* identification of gene coexpression modules to identify a cluster of four correlated gene modules strongly related to wheel-running behavior. These modules, numbered 4, 6, 11, and 24, are closely related (Fig. 5a). Each of these modules shows a strong relationship to running behavior, i.e. module membership correlates with individual genes’ relationships to running behavior (Fig. 5b). To visualize coexpression networks within these modules, we graphed genes as nodes and absolute correlations between genes of 0.85 or higher as edges (Fig. 5c). This visualization method identified a single large connected component. When we entered this coexpression network’s genes into DAVID, we found enrichment related to circadian rhythms, transcriptional regulation and protein phosphatase activity. The network contains genes of high neurobiological interest including Fosb, FosI2, Pdxd2 and a cluster of circadian-related genes.

Bridging the two modules, we found three genes with reciprocal coexpression: Baz1a, H11O, and Htr1b. The structure of the relationship is particularly noteworthy. The expression of H11O negatively correlates with both Htr1b and Baz1a expression, and Htr1b and Baz1a are positively correlated with one another. All three genes are strongly correlated with wheel-running behavior before wheels were blocked. As the Baz1a product has chromatin remodeling properties, the H11O product is a histone H1 subunit that would be remodeled by a chromatin remodeling factor and the Htr1b product is a serotonin receptor, we believe this coexpression bridge predicts a mechanistic relationship whereby chromatin remodeling factors affect chromatin state and the subsequent expression of serotonin receptors.

**LINCS connectivity mapping**
The list of 287 upregulated and 235 downregulated genes in high-runners compared with controls at FDR < 0.05 were queried against the LINCS database. The top 250 compounds for positive CSs are shown in Table S5. Considering only those compounds tested in neuronal cell lines, we found the protein kinase C δ (PKC δ) inhibitor, rottlerin, displayed a CS of +0.45. Considering compounds tested in multiple cell lines, we found the tyrosine kinase inhibitor Linifanib (CS: +0.45) and the delta-opioid receptor antagonist 7-benzylidenenaltrexone (BNTX, CS: +0.47). All three of these compounds represent candidate small molecules for further behavioral testing to determine the extent to which they might increase voluntary wheel-running behavior in mice. These compounds induced a pattern of gene expression in multiple cell lines that mimicked the striatal gene expression pattern found in high-runners relative to controls as indicated by the high positive CSs. Further, these compounds are known to affect monoamine signaling and have some literature on toxicity and efficacy in animal and human studies (Chen et al. 2016; Sofuoglu et al. 1993; Zhang et al. 2007).

**Discussion**
This is the first description of molecular pathways in the striatum associated with increased voluntary wheel-running behavior. Our study provides clues for how gene expression is altered in the striatum of mice highly motivated for aerobic exercise. The topic is of crucial importance, as levels of physical activity are currently declining and incidence of obesity and metabolic syndrome is rising at an alarming rate (Brownson et al. 2005). Our study is unique in many respects, including making use of an unprecedented, replicated, selective breeding experiment in which mice were bred for high voluntary wheel running for >60 generations (over two decades). The high-runner lines provide valuable insight never possible before into the genetic basis underlying increased motivation for physical activity. Before this study, it was known that high-runner mice displayed altered dopamine and serotonin signaling, and altered activation of the striatum associated with high motivation for wheel-running behavior (Claghorn et al. 2016; Kolb et al. 2013b; Rhodes & Garland 2003; Rhodes et al. 2001, 2003, 2005; Waters et al. 2013). However, the specific molecules that were altered by selection to affect the function of the striatum and increase motivation for running were not known. This study adds a new layer of mechanistic understanding regarding how gene expression is altered in the striatal reward circuit to increase motivation for exercise.

We interpreted the high-runner mice as highly motivated for wheel-running behavior based on a number of previous studies conducted by our group and others (Beiske & Garland 2007; Brene et al. 2007; Greenwood et al. 2011; Rhodes et al. 2005). Our interpretation is consistent with the idea that a high level of physical activity is perceived as rewarding in some animals, with variation in voluntary activity associated mostly with the reinforcement and perceived appetitive value of the activity. Consistent with this idea is that mice do not need to run to acquire any necessary resources (food, mating, etc.), yet they often choose to run at high levels (Lightfoot et al. 2004; Rhodes et al. 2005; Sherwin 1998; Werme et al. 2002). If running is rewarding, and we have uncovered pathways in the brain that are associated with high motivation for running reward, then our data may inform about molecular architecture of motivation in general, or in relation to other rewards. The full extent to which the molecular patterns described here generalize to other forms of increased motivation for rewards, such as increased motivation for food, sex or drugs of abuse is not known. When we compared genes differentially expressed at FDR < 0.10 to a dataset examining human gene expression related to cocaine and alcohol addiction (Farris et al. 2015), we found a modest relationship between the present differentially expressed gene set and genes differentially expressed in cocaine-abuse patient hippocampus (P = 0.043, hypergeometric test), but no relationship between the present differentially expressed gene set and genes differentially expressed in alcohol-abuse patient hippocampus (P = 0.371, hypergeometric test). When we compared our DEG list to another RNA-seq dataset comparing the effects of wheel running and alcohol consumption on striatal gene expression in C57BL/6J mice (Darlington et al. 2016), we found no significant overlap (for effects of running, P = 0.304; alcohol, P = 0.602; and interaction, P = 0.813.
Coexpression network analysis links chromatin state with monoamine receptor expression. (a) A cluster of coexpression modules with strong relationships with running behavior. (b) The relationship between the genes in these modules and running behaviors predicts the relationship between the genes in these modules and its module. (c) Visualizing the coexpression network shows multiple regions with strong previous neurobiological results. A bridge between network nuclei contains reciprocal relationships between the chromatin remodeling factor *Baz1a*, a histone H1 subunit *H1f0* and a metabotropic serotonin receptor *Htr1b*.
such as increased voluntary wheel running. Using a system genomics approach, our results suggest that novel gene expression pathways and networks mediate the high wheel-running phenotype, rather than nominating more obvious candidate genes (e.g., dopamine receptors, transporters and serotonin receptors) that are well-known to alter reward circuits. Instead, it appears the circuits are more likely to have been altered by a network of genes that affect chromatin in/or transcriptional states. Chromatin can exist in multiple functional states, either in a condensed state that prohibits gene expression or a relaxed state that facilitates gene expression. Thus, chromatin remodeling as a general biological function that emerges as a holistic property of the expression network as a whole is hypothesized to ultimately affect the function of reward circuitry, rather than individual gene effects occurring in isolation. This result describing the complexity in the regulatory changes has implications for association analyses that focus on candidate genes. Specifically, our study suggests that it is the gene expression networks that ultimately regulate individual differences in the development and function of the relevant brain circuits rather than individual components of the dopamine system (Flint & Munafo 2013).

Chromatin remodelers
One of the major discoveries of the current study was the central role of chromatin remodeling in orchestrating the gene expression profile of the striatum to support high motivation for voluntary running. The top DEG, the lncRNA 5031434O11Rik, which displayed lower expression in high-runner vs. control mice with no overlap between the two lines (Fig. 2a; FDR = 2.09 × 10^{-150}), is presumed to bind to the Setd7 mRNA as it overlaps the 5′ end of this gene. The Setd7 gene’s product is a lysine-histone-methyltransferase responsible for remodeling chromatin (Wilson et al. 2002). Setd7 itself displayed highly significant DEU event (FDR = 2.59 × 10^{-10}), a result we believe is related to the lncRNA’s differential expression. The putative mechanism is that this lncRNA binds to the 5′ end of the Setd7 transcript, acting as an antisense regulator that likely signals for the selective degradation of exons and results in the DEU observed for Setd7 (Qureshi & Mehler 2012). Future work is needed to sequence regulatory regions of the lncRNA 5031434O11Rik to determine whether selection favored certain alleles that ultimately decreased the expression of this chromatin regulator. In addition, future work with new long-read technologies could sequence the Setd7 gene in several high-runner and control mice to explicitly identify the different transcripts de novo, as there is only one transcript variant of Setd7 currently recognized in the Ensembl annotation. Given the role of Setd7 in remodeling chromatin, another potentially lucrative future direction would be to conduct chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) (Hon et al. 2009) and the related technique of batch isolation of tissue-specific chromatin for immunoprecipitation (BIT-S-ChIP) (Bonn et al. 2012) to determine the regions of the genome and sequences at those regions physically associated with Setd7 in multiple cell types.

Another indication that chromatin remodelers are likely targets for selection was the relationship between chromatin state and serotonin signaling as summarized in Fig. 5C. This graph shows strong negative correlations (Pearson’s r > 0.85) between expression of H110 and both Htr1b and Baz1a, whereas Baz1a and Htr1b are positively correlated with one another. All three genes are strongly correlated with wheel-running behavior before wheels were blocked. We interpret these results as evidence that differential expression of Baz1a gene product contributes to the remodeling of the chromatin in such a way that causes decreased expression of H110 histone subunit (Th’ng et al. 2005). More specifically, we imagine that the resulting altered chromatin and reduced histone H1 increases expression of Htr1b serotonin receptor in the striatum, which then increases motivation for running. These results suggest it may be profitable to sequence regulatory regions of the Baz1a gene to look for variants associated with selection.

It is particularly notable that the Baz1a gene product physically interacts with Smarca5 and other SWI/SNF proteins. Smarca4, a closely related SWI/SNF protein, was implicated in regulating differential hippocampal gene expression in a related strain derived from one of the high-runner lines (line 6) (Saul et al. 2012). Our group has also documented genomic variants in Smarca4 in the high-runner strain derived from line 6 that causally contribute to increased physical activity in the starting population, Hsd:ICR (unpublished data). Taken together, these data suggest a causal link between allelic variation at Smarca4, which results in non-synonymous substitution and altered protein product. The story would be as follows. A structural polymorphism in the SMARCA4 protein causes differential interaction with BAZ1A protein, altering the distribution of the H1F0 histone subunit protein and thereby creating a chromatin state conducive for increased expression of the serotonin receptor. Experiments directly assaying chromatin state, such as ChIP-Seq on acetyl marks of lysine 27 of histone H3 subunits (H3K27Ac; Hon et al. 2009) are a natural extension of the current work.

Cell-to-cell signaling and neurodevelopment
We discovered a strong difference in exon usage between high-runner vs. control mice in the Golgi signaling protein Tmed5, necessary for the secretion of Wnt ligands (Buechling et al. 2011). Unlike Setd7, Tmed5 has six known splice variants, and three of them showed large differences in expression, with one transcript more common in control and two more common in high-runner lines. The two transcripts that displayed increased expression in high-runner mice relative to control retained the luminal, but not the transmembrane or the cytoplasmic domains. Thus, we hypothesize that the DEU in high-runner mice results in a protein that acts antagonistically to and decreases the function of the canonical splice variant of this Golgi trafficking protein. Taken together, these results suggest that selective breeding may have altered transmembrane trafficking genes, which likely influence cell-to-cell signaling and neurodevelopment through altering secretion of Wnt ligands.

Mtds7a was highly differentially expressed, displaying significantly reduced expression in high-runner lines relative
to controls. This gene is not in the network shown in Fig. 5C and there is little known about the closest human homologue, MFSD7. Major facilitator superfamily domain genes are known to be related to the blood–brain barrier (Ben-Zvi et al. 2014), so we speculate that differential expression of this gene reflects composition of the blood–brain barrier, potentially affecting the transport of molecules from the blood to the brain and thereby affecting function. However, the specific molecules that would be affected remains unclear and would require further exploration.

**Receptors and chemical neurotransmission**  
Gpr3, a gene encoding an orphan G-protein-coupled receptor involved in signal transduction, and the serotonin receptor, Htr1b, display expression levels that were strongly positively correlated with levels of wheel running before wheels were blocked (Fig. 4a,c). Gpr3 is at the top of the gene network correlated with expression of Baz1a-Htr1b chromatin remodeling story described above (Fig. 5c). These genes are highly correlated with Fosl2 and other transcription factors. Hence, we hypothesize that Gpr3 and Htr1b are regulated through transcription factors and chromatin remodellers shown in Fig. 5c. Gpr3 is of particular interest; a novel pharmacological manipulation, AF64394, has recently been established to act as an inverse agonist of this receptor (Jensen et al. 2014). Based on the observed positive correlation between expression of Gpr3 and levels of voluntary running (before wheels were blocked), we hypothesize that treatment with this inverse agonist could decrease motivation, while treatment with an agonist could increase motivation for running. Recent papers have associated expression of Gpr3 with deposition of amyloid beta in Alzheimer’s disease (Huang et al. 2015). A recent paper found that Gpr3 knockout (Gpr3<sup>−/−</sup>) reduces monoamine content in the hippocampus, hypothalamus and frontal cortex along with increasing behavioral reactivity to stress (Valverde et al. 2009). Our data support the idea that GPR3 may represent a useful target in the modulation of voluntary physical activity and could constitute a novel and promising pharmacological target for increasing motivation for exercise.

The mature HTR1B and the GPR3 receptors would likely be on the membranes of GABA neurons, as this is the major neuronal type in the striatum (Tepper et al. 2004). These same GABA neurons would likely display dopamine receptors, and glutamine reuptake transporters on the cell surfaces. Expression of Slc38a2, a neuronal glutamine reuptake gene, was highly differentially expressed between high-runner and control lines and highly correlated with levels of wheel running (Fig. 4b). A related gene, Slc38a9, is in the network (Fig. 5c) away from the Baz1a-Htr1b-Gpr3 bridge. Astrocytes uptake glutamate and GABA, convert them to glutamine and transport glutamine back into the neuron via members of the Slc38a family, so increased expression of these glutamine transporters could indirectly reflect amount of glutamate or GABA, or could directly reflect altered recycling of these neurotransmitters to affect function of the striatal circuit (Gonzalez-Gonzalez et al. 2005; Schioto et al. 2013).

Gak, cyclin G-associated kinase, is not in the network shown in Fig. 5c, but it was highly differentially expressed between high-runner and control lines (2B). Gak is a serine-associated kinase involved in clathrin-mediated endocytosis (Lee et al. 2006). Increased expression of this protein could affect synaptic transmission by increasing neurotransmitter vesicle endocytosis. The large differential in Gak gene expression warrants further exploration of the role that this protein could have in increasing motivation for exercise.

**Candidate pharmaceuticals to increase motivation for exercise**  
Future studies will explore the truly novel concept of whether pharmacological compounds may be used to increase motivation for voluntary exercise. The LINCS tool generated many compounds which may theoretically achieve this aim (see Table S5). When considering CSs generated only from neuronal cell lines, the compound with the second highest positive CS was the PKC δ inhibitor, rottlerin. Rottlerin protects against striatal dopamine depletion in animal and cell models of Parkinson’s disease (Zhang et al. 2007). This makes rottlerin particularly relevant as altered dopamine signaling in the striatum is implicated in the heightened activity of the high-runner lines (Rhodes & Garland 2003; Rhodes et al. 2001, 2003, 2005; Waters et al. 2013).

Considering CSs generated from all the cell lines in the database, we found that the receptor tyrosine kinase (RTK) inhibitor, Linifanib (Chen et al. 2016), also displayed a high positive CS. The RTKs are high-affinity cell surface receptors for peptide growth factors, cytokines and hormones. They are known to play a critical role in neuron development and differentiation (Cadena & Gill 1992), but they are also widely expressed throughout the adult brain (Kotecha et al. 2002). In the hippocampus, they are known to affect synaptic strength (Levine et al. 1995) and to play a key role in the signaling cascade that connects stimulation of dopamine D2 receptors to altered glutamate receptor function (Kotecha et al. 2002). If RTKs play a similar role in connecting dopamine and glutamate signaling in the striatum, then they could contribute to altered exercise reward and motivation in the high-runner lines.

Another compound identified by LINCS as potentially efficacious for enhancing voluntary activity is BNTX, a selective delta 1-opioid receptor antagonist (Sofuoglu et al. 1993). Endogenous opioids have long been hypothesized to play a role in mediating the euphoria derived from voluntary exercise (Boecker et al. 2008). However, the euphoric effect of opioids is hypothesized to be mediated mainly via mu-opioid receptors. The role of delta-opioid receptors is less well understood. Recent studies suggest that selective delta-opioid receptor agonists have opposite effects as mu agonists on electrophysiological responses to somatosensory stimulation (Yokota et al. 2016) and selective knockouts of either mu or delta receptors result in opposite emotional behavioral responses (Filliol et al. 2000). Consistent with the hypothesis that blockade of delta-opioid receptors could increase motivation for exercise is that mice lacking delta-opioid receptors have been reported to be hyperactive (Filliol et al. 2000). Delta-opioid receptors have been hypothesized to inhibit striatal function (Le Merrer et al. 2013). Hence, one hypothesis for how delta receptor antagonist
could increase motivation for exercise is by releasing inhibition of opioid reward in the striatum and thereby increasing the potency of activity-induced euphoria. Our previous study reported no role for opioid signaling in the increased running in the high-runner lines (Li et al. 2004), but the opioid antagonists we used, naloxone and naltrexone, have much higher affinity for the mu-opioid receptor than delta- or kappa-opioid receptors (Emmerson et al. 1994; Raynor et al. 1994). Further, the euphoria associated with increased running could be mediated in part via cannabinoid signaling (Raichlen et al. 2013), which is known to functionally interact with opioid signaling (Miller et al. 2015; Vigano et al. 2005). This system has recently been implicated in high running behavior in humans and other mammals (Raichlen et al. 2012) and displays altered pharmacology in the high-runner lines (Keeney et al. 2008, 2012).

**Limitations**

Gene expression was only sampled at a single time-point in mice that were in the blocked-wheel condition. Previous work showed strong increases in c-Fos in cells in the striatum when mice were prevented from running but not when they were free to run, thus indicating an acute genomic response in the striatum when mice are prevented from running on their wheels (Rhodes et al. 2003). We interpreted this response as related to an emotional state of high motivation, or withdrawal from running, all related to increased motivation for running. In this study, the fos genes and those correlated with them may reflect an acute running withdrawal response (see also Kolb et al. 2013a; Malisch et al. 2009). However, because we only studied mice in the blocked condition, we cannot distinguish acute changes in gene expression resulting from blocking running from chronic changes in gene expression that persist or remain unchanged from the blocking treatment. Also note that both acute and chronic changes in gene expression could be causal, or consequential (i.e. result from the physical activity rather than cause it), although the previous study established that the c-Fos response was not related to acute differences in physical activity because levels were not correlated with running in the free-running animals (Rhodes et al. 2003).

Ultimately, we want to find the genetic polymorphisms in the high-runner lines that lead to their increased motivation to run. This is a more challenging undertaking. All of our gene expression results are correlational, and do not imply causation. Genetic polymorphisms that explain the differential expression of the key transcriptional regulators described herein would provide a causal link. A few quantitative trait loci (QTL) studies have been published focusing on the high-running phenotype, but they identified large chromosomal regions rather than single genes, and others focused on a muscle phenotype rather than the increased voluntary wheel running. We note that 76 DEGs in our study between high-runner and control fell in the QTL regions from table 3 of Nehrenberg et al. (2010), but this was not a significant enrichment, lending no additional support that genetic polymorphisms in these genes may contribute to the increased wheel running. In addition, we made one causal connection with the structural polymorphism in the Smarca4 gene previously identified as correlated with levels of physical activity in the founding strain (unpublished data), and interacting with Baz1a network described above (Saul et al. 2012). However, the gene expression data collected here suggest there could be many other places in the genome where alterations may have taken place to create the gene expression profile we observed. This includes IncRNA 5031434O11Rik and the Setd7 chromatin remodeler that need to be explored because they could open new understanding of how to increase motivation for exercise.

**Conclusions**

Results from this study advance our understanding of the neurogenetic underpinnings of increased voluntary wheel-running behavior in replicate lines of selectively bred mice. Our findings bridge chromatin structure with neural signaling by Gpr3 and Htr2b. The results imply a strong role for epigenetic control of neural signaling molecules in the regulation of motivation for exercise. Future work will examine this hypothesis directly by assessing the chromatin state through direct measurement, and in response to putative drugs intended to increase motivation for exercise.

**References**


Striatal transcriptome of mice born to run


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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

Appendix S1: Analysis summary and results of hypergeometric overlap test for overlap with previous data on alcohol and cocaine DEGs.
Table S1: Full differential expression results for multiple comparisons.
Table S2: Full WGCNA reporting, which includes measures of module fitting and robustness, module eigengene significance for selection and running and a report of all genes and their modules within the WGCNA.
Table S3: Complete DAVID results organized by tab into enrichment for genes upregulated (UP), genes downregulated (DOWN) and genes both upregulated and downregulated (BOTH).
Table S4: Results at FDR < 0.05 for DEXSeq DEU analysis.
Table S5: Results of LINCS analysis.
Figure S1: Diagnostic plots for the RNA-Seq dataset. (a) First two principal components of the expression in the RNA-Seq dataset shows no apparent pattern related to any of the selected lines. (b) and (c) Box-and-whiskers plots for RLE values from each sample for raw expression (b) and TMM-normalized expression (c) shows that while there was moderate variation in sample medians before TMM normalization, the normalization algorithm adequately addressed these differences. (d and e) Histograms for the distribution of P-values for the analysis on selection (d) and on running for 2 days before animals were blocked (e). Both P-value distributions are flat with the expected peak near 0.
Figure S2: Plot showing mean adjacency inside of all modules from the WGCNA compared with random WGCNA results with modules of the same size. Except for module 0, which holds genes that could not be clustered, all mean adjacency values are far higher than for random WGCNA results, indicating that the WGCNA found very good coexpression modules.