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Mesolimbic transcriptional response to hedonic substitution of voluntary exercise and voluntary ethanol consumption

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1. Introduction

Abuse of alcohol is a leading cause of preventable disease and death worldwide, affecting an estimated 76.3 million people [1]. Extensive research is being conducted on the development of alcohol use disorders, and a number of candidate genes have shown association with alcohol use [2]. Ethanol interacts with a variety of subcellular components comprising many of the known neurotransmitter systems including the mesolimbic dopaminergic pathway [3–5]. It has been proposed that a common pathway exists for addiction, and cross-tolerance between drugs of abuse, as well as co-abuse has been observed [5].

McMillan (1978) was the first to report the behavioral interaction of exercise and ethanol [6,7]. Since then, several groups have shown that access to exercise can influence voluntary ethanol intake [8–10]. Recent work in our laboratory supported the hypothesis that wheel-running may influence the reinforcing effects of ethanol [11]. This concept of hedonic substitution has been implemented in exercise intervention programs for humans consuming high quantities of ethanol [12–16]. Furthermore, the effects of exercise may not be limited to ethanol, and could be extended to amphetamine [17], nicotine [18] and cocaine [19].

While there is strong evidence that voluntary exercise can influence the consumption of ethanol, the mechanisms responsible for this interaction remain unclear. The mesolimbic dopaminergic (DA) pathway has been implicated in both ethanol consumption and exercise behaviors [3,20]. Both exercise and ethanol consumption acutely induce DA release in the striatum [20–23]. The mesolimbic DA pathway is composed of DA neurons originating in two sub-regions of the midbrain: substantia nigra (SN) and ventral tegmental area (VTA). These neurons project to the striatum—caudate-putamen and nucleus accumbens—as well as to regions of the frontal cortex. Also important is the hippocampus, which modulates the role of the striatum based on contextual learning. We examined the gene expression of six genes important in regulating this pathway which have also been previously associated with exercise and/or ethanol consumption. Tyrosine hydroxylase (*Th*), the rate-limiting enzyme in the DA synthesis pathway, has been shown to be up-regulated in certain sub-regions of the VTA of rats exposed to exercise [24], as well as in response to acute ethanol *in vivo* [25].

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We also examined two genes involved in DA release and reuptake. Solute carrier family 18 member a2 (*Slc18a2*) produces the vesicular monoamine transporter which packages cytosolic DA into synaptic vesicles, facilitating DA release. Solute carrier family 6 member a3 (*Slc6a3*) codes for the dopamine active transporter, which is responsible for the reuptake of synaptic DA. Neither of these genes has been studied at the neurophysiological level for response to exercise or alcohol. Variations in the human *SLC18A2* and *SLC6A3* genes have been associated with alcohol disorders [26–28]. There is conflicting evidence that female *Slc18a2* knockout mice drink less ethanol [29,30], but *Slc6a3* knockout mice do not show differences in voluntary alcohol consumption [29]. In addition, we consider the two genes that code for DA receptors, dopamine receptor d1 (*Drd1a*) and dopamine receptor d2 (*Drd2*). Increased expression of *Drd1a* has been observed in ethanol-dependent mice [31], while lower *Drd1a* expression was found in high-running strains of mice [32,33]. Over-expressing *Drd2* reduced ethanol consumption in ethanol preferring rats [34,35], and high-alcohol preferring mice express striatal *Drd2* at lower levels than low-alcohol preferring mice [36]. However, *Drd2* expression was significantly less in high-running mice [33]. Brain derived neurotrophic factor (*Bdnf*), the final gene in which we were interested, was selected based on its general role in promoting cell survival and proliferation. *Bdnf* expression has been shown in several studies to be increased after exercise [20,37,38]. Furthermore, over-expression of *Bdnf* led to decreased ethanol consumption while *Bdnf* heterozygous knockouts had increased ethanol consumption [39]. Table 1 provides a summary of the expression patterns, functions, reasons for inclusion in the study, and references for these genes.

This study was designed with two aims. First we wanted to replicate the phenomenon of hedonic substitution, and second to investigate mesolimbic DA pathway gene expression plasticity in response to access to ethanol and wheel running that may account for some of the behavioral differences.

2. Methods

2.1 Statement on animal care

This study was conducted with approval from the Institutional Animal Care and Use Committee at the University of Colorado, Boulder (Boulder, Colorado) following guidelines established by the Office of Laboratory Animal Welfare. All possible measures were taken to minimize animal discomfort.

2.2 Animals

Animals were bred and housed at the Specific Pathogen Free facility, operated by the Institute for Behavioral Genetics at the University of Colorado, Boulder (Boulder, Colorado). Female C57BL/6J mice aged 60–90 days were used for these experiments. Animals were individually housed in polycarbonate cages (30.3 × 20.6 × 26 cm) on a 12-hour light/dark cycle with lights on at 7:00 AM. Room temperature was maintained between 23 and 24.5°C. All mice had *ad libitum* access to standard chow (Harlan Laboratories, Indianapolis, Indiana) and water. Animals were monitored daily and body weights were recorded every 4 days. Food was weighed every 4 days, on the same schedule as body weights.

2.3 Behavioral paradigm

Mice were tested using a previously established paradigm that lead to differences in ethanol consumption when given access to a free running wheel [11]. The four conditions (n=15/condition) included cages with 1) water only, 2) 1 bottle of water and 1 bottle of ethanol (two-bottle choice), 3) water and ethanol two-bottle choice with a running wheel, and 4)

water only with a running wheel. The protocol lasted 16 days. Mice housed with a running wheel (diameter 24.2cm, Harvard Apparatus, Holliston, Massachusetts) had 24-hour access to the wheel for all 16 days. Wheel revolutions were counted using a magnet and magnetic switch (Harvard Apparatus) and recorded daily. Mice housed with ethanol two-bottle choice progressed as follows: water only for days 1–3, 3% ethanol (v/v) for days 4–5, 7% ethanol for days 6–7, and 10% ethanol for days 8–16 (Table 2). The side of the cage the bottles were on was alternated every two days. Individual consumption of water and ethanol (if applicable) were recorded daily. On day 16 during the second hour of the light cycle, mice were sacrificed by cervical dislocation. Groups of 5 mice were staggered to start the protocol every 2 days so as to minimize the variation in tissue collection times on day 16. Daily measurements of wheel revolutions (1 day each for 4 mice), water (1 day for 1 mouse) and ethanol consumption (1 day each for 4 mice) are missing due to sporadic equipment failure (i.e. switches detecting wheel magnets could be bumped out of alignment or fluid tubes could leak if stopper seal was not secured tight enough). These missing values were imputed as the average of the preceding and following days.

2.4 Saccharin control group

In addition, 10 mice were housed with two-bottle choice water and saccharin in two cage conditions (n=5/condition), either with or without wheel in cages described above. After water only for days 1–3, a 0.033% saccharin solution was added for days 4–16 [40,41]. This concentration was sufficient to produce approximately 95% preference in two-bottle choice. The side of the cage the bottles were on was alternated every two days and individual consumption of water and saccharin were recorded daily.

2.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Whole brains were removed and the midbrain, striatum, hippocampus, and cortex were dissected and stored in RNALater™ (Ambion, Foster City, California) at –20°C. Total RNA from dissected regions was extracted using EZNA Total RNA Kit II (Omega Bio-tek, Norcross, Georgia). Quality and quantity of RNA were determined by gel electrophoresis and NanoDrop™ spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts). A260/A280 was determined to be excellent in each case (>1.8). Total mRNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California). For real-time quantitative PCR, we used Taqman™ primers and probes (Applied Biosystems) for the following genes: *Bdnf* (Mm04230607_s1), *Drd1a* (Mm01353211_m1), *Drd2* (Mm00438545_m1), *Th* (Mm00447557_m1), *Slc6a3* (Mm00438388_m1), and *Slc18a2* (Mm00553058_m1). Endogenous genes *Gapdh* (4352339E) and *Actnb* (4352341E) were used as controls. Real-time quantitative PCRs were performed using an ABI 7900HT (Applied Biosystems) running Sequence Detection Systems software (SDS v2.3, Applied Biosystems). All target genes were normalized using the $2^{-\Delta\Delta C_t}$ method [42,43].

2.6 in situ hybridization (ISH)

Whole brains were removed and flash frozen in isopentane on dry ice and stored at –70°C. Brains were sectioned coronally into 14 micron slices using a cryostat (Leica, Wetzlar, Germany), thaw mounted on poly-L-lysine coated glass slides (ThermoFisher Scientific) and stored at –70°C. We used a previously established method for *in situ* hybridization of radiolabeled antisense riboprobes [44]. Briefly, probes were transcribed *in vitro* with ^{35}S -UTP (PerkinElmer, Waltham, Massachusetts) as the sole source of UTP. Constructs for three genes, *Drd1a*, *Bdnf*, and *Slc18a2* were cloned into pT3T7 transcription vectors, were acquired from ThermoFisher Scientific: *Drd1a* – EMM1032-613237 (600bp), *Bdnf* – EMM1032-607279 (800bp), *Slc18a2* – EMM1032-591860 (1500bp). All vectors were

linearized with EcoRI (New England Biolabs, Ipswich, Massachusetts) and transcribed using T3 RNA polymerase (Promega, Fitchburg, Wisconsin). Constructs for other three genes, *Th*, *Slc6a3*, and *Drd2* were cloned from cDNA used for the RT-PCR experiment. Primers (www.idtdna.com) were designed using Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) software, and a T7 binding site was added to each reverse primer. Primer sequences were as follows: *Th* forward (GCCGTCTCAGAGCAGGATAC) and reverse (GAAATTAATACGACTCACTATAGGACCAGGGAACCTTGTCCTCT); *Slc6a3* forward (GAGGTTCAAGAGCGGGAGAC) and reverse (GAAATTAATACGACTCACTATAGGGATCCACACCACCTTCCCTG); and *Drd2* forward (CTTTGCAGACCACCAAC) and reverse (GAAATTAATACGACTCACTATAGGGTGGGTACAGTTGCCCTTGA). Twenty five PCR cycles were used to generate clones. Transcription was carried out using T7 RNA polymerase (Promega). Hybridizations were performed within 1 day of transcription.

On the day of hybridization, after warming to room temperature, tissue was first fixed with a 4% paraformaldehyde solution (15min), rinsed with 1x phosphate buffered saline (3×5min), then rinsed with 0.1M TEA (2min). Next the tissue was acetylated with 0.25% acetic anhydride in 0.1M TEA (15min) and then dehydrated in graded ethanol solutions of 50%, 70%, 95%, 100% and 100% (3min each). Radiolabeled riboprobes were diluted in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 300mM NaCl, 10mM Tris, 1mM EDTA, and 1x Denhardt's solution, and ~100μL were pipetted onto a 24mm × 60mm coverslip, then placed upside down covering tissue. Coverslips were sealed to slides using DPX mountant (Sigma-Aldrich, St. Louis, Missouri). Tissue sections were hybridized with riboprobe solution for 16 hours at 60°C. After hybridization, tissue section slides were washed with 4x saline sodium citrate (SSC) before being treated with RNase A (20μg/mL) for 1 hour at 37°C. Then tissue sections were desalted by incubation in graded SSC solutions (all with 1mM DTT) to a final stringency of 0.1xSSC at 65°C. Finally, sections were dehydrated with graded ethanol solutions, dried, and exposed to PhosphoScreens (Packard, Meriden, Connecticut) for at least 1 week. Slides for every mouse for each riboprobe were assayed at the same time to allow for direct comparisons between mice.

In order to relate the intensity of each screen image to a relative measure of tissue radioactivity, tissue standards containing known amounts of ³⁵S were exposed along with tissue on each film. Tissue standards were prepared by mixing measured amounts of isotope with a homogenate prepared from whole brain. Actual concentrations of radioactivity were measured in weighted aliquots. The ³⁵S standards contained from 0 to 25 nCi/mg. Ten standards were used for each isotope, and were used to construct standard curves relating optical density and a measure of radioactivity (counts per minute per mg).

Exposed PhosphoScreens were imaged with a Cyclone PhosphoImage reader (Packard), and image. tif files (600 dpi) were imported into the OptiQuant analysis suite (Packard). Slides were de-identified and brain regions of interest were circled as well as background. At least 3, and as many as 20 measurements were taken from each animal, and the values obtained were averaged for each animal.

2.7 Statistical analyses

A one-way repeated measures ANOVA was used to identify group differences in alcohol consumption (runners vs. non-runners). A one-way repeated measures ANOVA was used to identify group differences in daily wheel revolutions (drinkers vs. non-drinkers). A two-way repeated measures ANOVA was used to determine group differences in body weight (2×2 drinkers vs. non-drinkers and runners vs. non-runners). For repeated measures ANOVAs, missing daily values were imputed from the average of the previous and following days' values. A two-way ANOVA was used to determine group differences due to cage and fluid

for food consumption data and for gene expression data (2×2, drinkers vs. non-drinkers and runners vs. non-runners). Average ΔC_t values for each mouse were used for RT-PCR. Average CPM/mg values for each mouse were used for *in situ* hybridization data. Repeated measures ANOVAs were calculated using SPSS v20, two-way ANOVAs were calculated using R v2.15.2 (www.r-project.org).

3. Results

3.1 Mice

Body weights increased over the course of 16 days ($F_{3,168}=29.32$, $p<0.001$) but there were no main effects of ethanol or running. There was a slight difference in the amount of food consumed, with significant main effects observed for both ethanol and a running wheel. Mice that had access to ethanol consumed less food than mice that only had access to water (3.52 ± 0.09 g/day vs. 3.85 ± 0.08 g/day, respectively; $F_{1,56}=8.393$, $p<0.01$). Mice with access to a running wheel consumed more food than mice housed in empty cage (3.83 ± 0.09 g/day vs. 3.54 ± 0.08 g/day, respectively, $F_{1,56}=6.811$, $p<0.05$).

3.2 Voluntary running and ethanol consumption

As expected, mice ran a considerable distance each day, averaging 7144 revolutions per day, equaling 5431 meters per day. There was a slight increase in daily revolutions over the course of 16 days (Figure 1, $F_{15,420}=2.8$, $p<0.001$) There was no significant difference in number of revolutions between mice with access to water only and mice with access to ethanol.

Mice with access to running wheel significantly consumed (g/kg; $F_{1,28}=11.6$, $p<0.01$) and preferred ($F_{1,28}=30.7$, $p<0.001$) less ethanol than mice housed in an empty cage over the course of 16 days (Figure 2a and 2b).

3.3 Saccharin control experiment

In the ten mice in the saccharin control experiment there was no significant change in body weight over the 16 days, nor was there any effect of access to a running wheel. Access to a running wheel did not significantly change saccharin consumption as measured by milligrams saccharin per kilogram body weight or as a saccharin preference ratio (data not shown).

3.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

There were no significant differences in gene expression between groups for *Th*, *Drd2*, and *Slc6a3*. In addition, there were no differences in gene expression for *Drd1a* when measured in the cortex. In the midbrain, there was a main effect of ethanol availability on expression of *Slc18a2* (Figure 3a, $F_{1,16}=18.9$, $p<0.001$), ethanol-consuming mice showing increased expression compared to the group that only had access to water. In the striatum, there was a main effect of ethanol availability on the expression of *Drd1a* (Figure 3b, $F_{1,16}=6.9$, $p<0.05$), with ethanol-consuming mice showing decreased expression levels. In the hippocampus, there were significant main effects of running wheel availability ($F_{1,17}=6.3$, $p<0.05$) and ethanol availability ($F_{1,17}=5.5$, $p<0.05$) on *Bdnf* expression (Figure 3c). Running mice had increased expression of *Bdnf*, while ethanol-drinking mice had decreased expression.

3.5 *in situ* hybridization (ISH)

We measured gene expression using *in situ* hybridization (ISH) to confirm the results from qRT-PCR. There were no differences in gene expression in examined regions between

groups for *Th*, *Drd2*, and *Slc6a3*. There were differences in expression for midbrain *Slc18a2* and striatal *Drd1a* when measured using ISH. Contrary to expression levels detected through qRT-PCR, there was an ethanol x running wheel interaction effect on the expression of *Slc18a2* in both midbrain sub-regions: the substantia nigra ($F_{1,18}=5.2$, $p<0.05$) and the ventral tegmental area ($F_{1,18}=5.6$, $p<0.05$, Figures 4a and 4b). The interaction effect was as follows: in empty cages, ethanol-drinking mice had increased expression of *Slc18a2* compared to water-only mice, while in cages with running wheels; ethanol-drinking mice had decreased expression of *Slc18a2*. In the striatum, there was a main effect of running wheel availability on expression of *Drd1a* (Figure 4c). Mice with access to running wheels showed decreased expression *Drd1a* compared to mice without access ($F_{1,20}=7.0$, $p<0.05$). There was no effect of availability of ethanol on *Drd1a* expression. In concordance with expression levels detected through qRT-PCR, there were significant main effects of access to running wheel ($F_{1,20}=4.4$, $p<0.05$) and access to ethanol ($F_{1,20}=7.7$, $p<0.05$) on expression of *Bdnf* in the dentate gyrus of the hippocampus (Figure 4d). Access to a running wheel increased expression, while access to ethanol decreased expression. There was no significant interaction effect. Representative images from each ISH assay are shown in Figure 5.

4. Discussion

4.1 Evidence for hedonic substitution

This study provides additional evidence for the behavioral interaction between voluntary exercise and ethanol consumption. Using a similar protocol as in Ehringer et al. (2009), we observed decreased ethanol consumption and preference in female C57BL/6J mice than in their non-running counterparts [11]. Ehringer (2009) demonstrated that the substitution effect was due specifically to a running wheel, and not to a locked wheel, at least in females. Further, they went on to show that ethanol metabolism was unchanged due to running. The current study shows running on an exercise wheel failed to reduce consumption and preference for a saccharin solution, suggesting that the effects of exercise may not be sufficient to alter all rewarding behaviors.

Contrary to Ehringer et al. (2009), which studied hedonic substitution in both sexes, the current study only examined females. Although this limits the interpretation of the findings, female C57BL/6J mice run more and drink more than their male counterparts, and in this preliminary search for genes involved in hedonic substitution, it was important that the substitution effect was most robust, as is more consistently observed in females. Furthermore, while there may be concern about effects of the estrous cycle, it is likely that our sample size and the length of the study wash out any effects. Sixteen days encompasses 4–5 complete cycles, therefore any effects across our group of mice should only add random noise. Unfortunately, the invasive nature of monitoring the estrous cycle [45] would have fundamentally, and adversely, affected the behavioral paradigm.

4.2 Transcriptional changes in mesolimbic reward pathway

In addition to providing evidence for the behavioral interaction, this study attempted to elucidate the underlying molecular basis for hedonic substitution, utilizing measures of gene expression. Although exercise has been and is currently being used as a behavioral intervention for alcohol use disorders, a more thorough understanding of the mechanisms involved could provide a framework for more effective interventions. The implication of the mesolimbic DA pathway in the regulation of voluntary exercise and ethanol consumption provided a foundation for hypothesizing which genes could play a role. We selected genes based on their role in modulating this pathway, in addition to prior implications in exercise and ethanol behaviors.

We examined the expression of six genes, all actors in the mesolimbic DA pathway, first by quantitative real-time PCR, then by *in situ* hybridization (ISH). In the midbrain we were unable to detect changes in expression of *Th*, *Drd2*, and *Slc6a3*. Although several groups have shown an increase in *Th* in response to exercise and ethanol, protocol and organism differences may explain the discrepancy in findings. Greenwood (2011) saw differences in the caudal third of the VTA of male Fischer 344 rats after 6 weeks of running, with no differences in the mid and rostral portion, and no differences reported for SN [24]. Thus, in this case there are several possible explanations for differences with our study. First, sex differences are often seen in behavioral paradigms and that study included only males, while ours focused on females. Second, Greenwood studied rats, whereas we are tested mice. Third, in the context of the larger rat brain, it is easier to dissect out specific regions as was performed to distinguish between caudal, mid, and rostral sections of the VTA. In addition, and supporting our finding, Nascimento et al. (2011) did not see an increase in *Th* expression in the VTA of rats after 8 weeks of running compared to untrained control rats [46]. Other *in vitro* studies observed increases in *Th* in response to acute ethanol administration [25,47], but our protocol is a voluntary unlimited continuous access, which is quite different than acute exposure.

With regard to *Drd2*, the lack of differences observed in our study is not entirely inconsistent with previous papers that have implicated a role of this gene in both alcohol and exercise. Polymorphisms in *Drd2*, as well as changes in expression have been implicated in both behaviors. Specifically, polymorphisms in the human gene *DRD2* have been associated with alcohol dependence [48]. Striatal *Drd2* expression has been shown to be less in high running mice [33] or the same in different strains of mice shown to differ in baseline running [32]. However, overexpression of *Drd2* has been associated with decreased ethanol consumption in rats [34,35]. Similarly, high alcohol preferring mice express less *Drd2* than low alcohol preferring mice [36]. It seemed plausible that running may induce *Drd2* expression, thereby decreasing ethanol consumption, although our results do not support this hypothesis. Additional studies are needed to clarify possible regional brain differences and/or temporal effects than may account for the inconclusive findings.

Our failure to find differences in *Slc6a3* is somewhat consistent with previous reports showing knockout mice have similar ethanol consumption, at least in females, as their wild-type controls [29]. However, polymorphisms in *SLC6A3* have been associated with alcohol use disorders in humans [26], so there was justification to examine it. To our knowledge, this gene has not been investigated in the context of exercise. Therefore, it remains unclear whether the expression of *Slc6a3* affects voluntary ethanol or exercise behavior; one possibility is that any effects it exerts may be mediated through a different region of the brain.

In the midbrain, mRNA expression measured using qRT-PCR assays of *Slc18a2* appeared to increase in response to consumption of ethanol. However, when looking at more fine-grained patterns in the VTA or SN using the *in situ* technique, the main effect of ethanol was abolished, and we observed an interaction effect. In the absence of a running wheel, ethanol led to increased expression; while in running mice, ethanol led to decreased expression. Other midbrain regions expressing *Slc18a2*, such as the raphe nuclei in the caudal midbrain, may account for some of these differences, and highlight the importance of more targeted regional selections. The interaction effect observed in the VTA and SN is particularly interesting, and we speculate that in the absence of running, there could be increased DA release due to ethanol, which facilitates the need for higher expression of *Slc18a2*. This could be tested in future studies. Two studies have associated polymorphisms in the human gene *SLC18A2* with alcohol use disorders [27,28], and two studies have examined ethanol behavior with *Slc18a2* knockout mice [29,30]. In female mice, the two studies show

conflicting results, with the effect of heterozygous deletion of *Slc18a2* having no effect [29] or decreasing ethanol preference [30]. Our study adds support to the idea that expression levels of *Slc18a2*, not just polymorphisms, are important for ethanol-related behaviors.

In the striatum, expression of *Drd1a* showed different patterns of response dependent on the method of detection. In the qRT-PCR experiments, *Drd1a* showed decreased expression in response to ethanol, while this decrease was observed in the wheel running condition for *in situ* assays. The mice in this study were voluntarily drinking ethanol, and therefore not ethanol-dependent, however Contet et al. (2011) observed increased *Drd1a* expression in ethanol-dependent mice [31]. Our observation of reduced expression measured by ISH is consistent with work by Knab et al. (2009), who observed lower baseline *Drd1a* expression in a high-running strain of mice compared to a low-running strain [32]. Thus, there remains a high interest in this gene for its role in these two behaviors, but further studies should address specificity of alcohol exposure, possible temporal effects, and other brain regions.

Our data, consistent using both qRT-PCR and ISH, show that 16 days of voluntary exercise increases hippocampal *Bdnf* expression and 16 days of voluntary ethanol consumption decreases expression, which suggests changes in neuronal structure and neurogenesis. The hippocampus provides contextual information to the striatum based on prior associated experiences [3]. In one of the few studies examining the influence of exercise on ethanol behaviors, Leasure and Nixon (2009) demonstrated exercise's ability to protect hippocampal cells from the effects of binge ethanol consumption [49]. These findings complement other work showing the ability of exercise to initiate neurogenesis in the hippocampus [50,51], as well as other studies showing that changes in *Bdnf* expression result in changes in ethanol behaviors [reviews 39,52]. Alterations in the hippocampus due to exercise may influence the signaling between the striatum and hippocampus, affecting the reward response to ethanol drinking.

Conclusions

These data reaffirm the hedonic substitution narrative, and make the first attempt at identifying the underlying genetic changes that occur to influence this interaction. Of the genes in which differences were observed, *Slc18a2* expression in the VTA and SN responds differently to ethanol depending on the presence or absence of a running wheel, and *Bdnf* expression in the hippocampus changes in response to both running and ethanol. *Drd1a* in the striatum may also be responsive to both running and ethanol. This suggests that multiple genes and brain regions are important in regulating hedonic substitution, and supports the idea that the mesolimbic DA pathway plays an important role. Future studies should focus on global gene expression to identify other genes as well as assessing whether observed mRNA changes correspond to similar protein changes. It will also be useful to expand this behavioral model to include testing whether running produces similar effects on voluntary consumption of other drugs of abuse.

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Highlights

- 16 days of wheel running decreased ethanol consumption, with no effect on saccharin consumption.
- Changes in expression of midbrain *Slc18a2* due to ethanol were dependent on running.
- Running and ethanol consumption had opposing effects on hippocampal *Bdnf* expression.
- Striatal expression of *Drd1a* was depressed by both running and ethanol.

Fig. 1.

Average daily running wheel revolutions for mice with water only and mice with two-bottle choice ethanol over 16 days. Average number of revolutions for water only mice (Circles, $n=15$, 7486 ± 590 revolutions/day) and for ethanol-drinking mice (X's, $n=15$, 6798 ± 584 revolutions/day). Mean $\pm$ SEM are reported.

Fig. 2.

Average daily ethanol consumption for mice with empty cage (Circles) and mice with access to a running wheel (X's) over 16 days. Ethanol consumption is shown as average amount of ethanol consumed per body weight (A) and as an ethanol preference ratio (B) defined as volume of ethanol fluid consumed divided by total fluid consumed. Ethanol concentrations (v/v) for each day are reported on the x-axis. Mean $\pm$ SEM are reported.

Fig. 3.

Relative mRNA expression levels as measured by real-time quantitative PCR for (A) *Slc18a2* (midbrain), (B) *Drd1a* (striatum), and (C) *Bdnf* (hippocampus). Main effects due to availability of ethanol were observed in midbrain *Slc18a2*, striatal *Drd1a*, and hippocampal *Bdnf*. Main effects due to availability of a running wheel were observed in hippocampal *Bdnf*. There were no significant interaction effects. Expression levels are shown as mean fold change $\pm$ SEM relative to sedentary/water only group for each gene. No significant differences were observed in other genes/regions.

Fig. 4.

Results of in situ hybridization showing mRNA expression levels for *Slc18a2* in midbrain subregions: (A) substantia nigra (SN) and (B) ventral tegmental area (VTA), *Drd1a* in (C) striatum (ST), and (D) *Bdnf* in hippocampus (HC). Significant interaction effects were observed in both midbrain regions for *Slc18a2*. Significant main effects due to availability of running wheel were observed in striatal *Drd1a* and in hippocampal *Bdnf*. A main effect due to availability of ethanol was observed in hippocampal *Bdnf*. Values shown (mean $\pm$ SEM) have been converted to counts per minute.

Fig. 5.

Representative images of each *in situ* hybridization assay. For *Th* (A), *Slc6a3* (B), and *Slc18a2* (C), both the substantia nigra (SN) and ventral tegmental area (VTA) were quantified. For *Drd1a* (D), the whole striatum (ST) was quantified. For *Drd2* (E and F), the ST (E), SN and VTA (F) were quantified. For *Bdnf* (G), the dentate gyrus of the hippocampus was quantified.

Table 1

List of genes assayed for expression, and relevant details.

Gene name	Translated protein	Brain expression	Function	Reason for inclusion	References
<i>Th</i>	Tyrosine hydroxylase	Midbrain (ventral tegmental area, substantia nigra), and Pons (locus coeruleus)	Rate-limiting enzyme in production of dopamine, hydroxylizes tyrosine into L- DOPA	Implicated in exercise and ethanol behaviors	[24-25,46-47]
<i>Slc18a2</i>	Vesicular monoamine transporter 2	Midbrain (ventral tegmental area, substantia nigra, raphe nuclei) and Pons (locus coeruleus)	Packaging of cytosolic dopamine into synaptic vesicles to facilitate release	SNPs associated with ethanol behavior, and with locomotor behavior. Knockout mice (+/-) drink more.	[27-30]
<i>Slc6a3</i>	Dopamine active transporter	Midbrain (ventral tegmental area and substantia nigra)	Reuptake of dopamine from the synapse	SNPs associated with ethanol behavior	[26,29]
<i>Drd1a</i>	Dopamine receptor D1	Striatum, cortex, olfactory tubercles, olfactory bulbs	G-protein coupled receptor - signaling cascade activates adenylyl cyclase	Implicated in exercise and ethanol behaviors	[31-33]
<i>Drd2</i>	Dopamine receptor D2	Midbrain, striatum, and cortex	G-protein coupled receptor - signaling cascade decreases adenylyl cyclase	Implicated in exercise and ethanol behaviors	[33-36,48]
<i>Bdnf</i>	Brain-derived neurotrophic factor	Many regions, but highly expressed in hippocampus	Nerve growth factor important for cell survival and proliferation	Increased after exercise, may play role in exercise neuroprotection from binge ethanol consumption	[20,37-39,52]

Table 2

2×2 Behavioral paradigm for wheel running exposure and ethanol consumption

	Days 1–3	Days 4–5	Days 6–7	Days 8–16
Running	Water only	3% ethanol & water	7% ethanol & water	10% ethanol & water
	Water only			
Sedentary	Water only	3% ethanol & water	7% ethanol & water	10% ethanol & water
	Water only			