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NEUROPLASTICITY, AXONAL GUIDANCE, AND MICRORNA GENES ARE ASSOCIATED WITH MORPHINE SELF-ADMINISTRATION BEHAVIOR

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Abstract

Neuroadaptations in the ventral striatum (VS) and ventral midbrain (VMB) following chronic opioid administration are thought to contribute to the pathogenesis and persistence of opiate addiction. In order to identify candidate genes involved in these neuroadaptations we utilized a behavior genetics strategy designed to associate contingent intravenous drug self-administration with specific patterns of gene expression in inbred mice differentially predisposed to the rewarding effects of morphine. In a yoked-control paradigm, C57BL/6J mice showed clear morphine-reinforced behavior whereas DBA/2J mice did not. Moreover, the yoked-control paradigm revealed the powerful consequences of self-administration versus passive administration at the level of gene expression. Morphine self-administration in the C57BL/6J mice uniquely up- or down-regulated 237 genes in the VS and 131 genes in the VMB. Interestingly, only a handful of the C57BL/6J self-administration genes (<3%) exhibited a similar expression pattern in the DBA/2J mice. Hence, specific sets of genes could be confidently assigned to regional effects of morphine in a contingent- and genotype-dependent manner. Bioinformatics analysis revealed that neuroplasticity, axonal guidance and microRNAs (miRNAs) were among the key themes associated with drug self-administration. Noteworthy were the primary miRNA genes *H19* and microRNA containing gene (*Mirg*), processed respectively to mature miRNAs miR-675 and miR-154, since they are prime candidates to mediate network-like changes in responses to chronic drug administration. These miRNAs have postulated roles in dopaminergic neuron differentiation and mu-opioid receptor regulation. The strategic approach designed to focus on reinforcement-associated genes provides new insight into the role of neuroplasticity pathways and miRNAs in drug addiction.

Keywords

Opioid; addiction; neuroadaptation; vulnerability; reward; microRNA; H19

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Introduction

Understanding the process that leads an individual from experimentation, to drug use and eventually to abuse is one of the most important questions in vulnerability research. The transition from drug use to dependence appears to be influenced more profoundly by heritable factors than the initial experimentation process (van den Bree et al., 1998; Kendler et al., 1999; Tsuang et al., 1999; Kendler et al., 2000), and opioid abuse appears to be somewhat unique in that it shows particularly high heritability that may have distinct genetic characteristics from other drugs of abuse (Merikangas et al., 1998; Tsuang et al., 2001). Individuals genetically predisposed to opioid abuse may inherit a neurochemical system that is vulnerable to molecular and cellular adaptations facilitating the transition from drug use to abuse (Nestler, 2001; Gerdeman et al., 2003; Kalivas and O'Brien, 2008).

The significant influence genotype has on the behavioral effects of opiates in the human population is substantiated in preclinical behavior genetics studies involving commonly available inbred rat and mouse strains. The consequences of chronic opioid exposure, such as analgesic tolerance, is significantly influenced by genotype (Kolesnikov et al., 1998; Crain and Shen, 2000; Kest et al., 2002), even when the dose of morphine is adjusted to account for differences in acute sensitivity (Tapocik et al., 2009). Potential quantitative and qualitative differences in drug self-administration have been reported as well. In particular, our laboratory has demonstrated operant opioid-reinforced behavior (Ambrosio et al., 1995; Elmer et al., 1995; Elmer et al., 1996; Elmer et al., 2010) and potentiation of brain stimulation reward (Elmer et al., 2005; Elmer et al., 2010) to be significantly influenced by genotype.

The specific neurobiological processes underlying the transition from occasional drug use to addiction are not well characterized. Abused drugs act on the reward system over time to induce molecular alterations to cellular pathways and thereby modify synaptic connections (Williams et al., 2001; Kauer and Malenka, 2007; Kalivas and O'Brien, 2008). Furthermore, genetic variation in the long-term neuroadaptations and synaptic plasticity that arise during the transition to drug addiction are likely represented by widespread differences in gene expression (McClung and Nestler, 2008; Maze et al., 2010). Genetically defined animal models, such as the C57BL/6J and DBA/2J inbred strains, provide a framework to explore these genetic influences (Tapocik et al., 2009; Elmer et al., 2010). The genetic backgrounds that give rise to anatomical and neurochemical differences between genotypes may explain the divergent behavioral and neuroadaptive responses to chronic morphine exposure.

We hypothesize that a specific set of gene expression changes are associated with vulnerability to drug-seeking behavior. To elucidate the genes and pathways involved in the transition to a regular drug self-administration habit, we exploited two inbred strains that differ significantly in the rewarding effects of morphine, large-scale gene expression profiles in two brain regions, and a yoked-control behavioral paradigm designed to filter out gene expression changes related to surgical procedures and passive drug exposure. The results provide insight into neuroadaptations that may contribute to the pathogenesis of opiate addiction.

Materials and Methods

Animals

Adult male C57BL/6J (B6) and DBA/2J (D2) mice (Jackson Laboratories, Bar Harbor, ME) 60–120 days old and weighing approximately 21–28 g at the start of the experiment were used. All animals were experimentally naive, housed in a temperature-controlled room (21°

C; 0700–1900 lights on), and given free access to chow and tap water during drug self-administration experimental procedures. D2 (N = 12) and B6 (N = 27) mice were randomly distributed into three different yoked conditions (Yoker, Yoked-morphine, Yoked-saline). The facilities were fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and the studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the NIH.

Apparatus & Water Reinforcement Training

Details pertaining to the self-administration apparatus and water reinforcement training can be found in Supplemental Materials and Methods.

Surgery

Following water training, subjects were surgically prepared with a catheter implanted in the jugular vein. Surgical procedures were performed under ketamine- (100 mg/kg, i.p.) and xylazine- (16 mg/kg, i.p.) induced anesthesia. Silastic tubing (0.012" i.d.) was implanted in the right jugular vein approximately to the level of the atrium. The catheter was passed subcutaneously and exited in the midscapular region. The catheter was connected to a tether and swivel system (Instech, Plymouth Meeting, PA, USA) attached to the skull with dental cement (DenMat, CA, USA). The liquid swivel system was mounted inside the self-administration chamber where the mouse remained for the duration of the experiment. The mice were allowed to recover from surgery for 7 days.

Intravenous drug self-administration

The self-administration experiment was run using a Yoked-control paradigm with three experimental groups (Yoker, Yoked-morphine, Yoked-saline). After recovery from surgery, the Yokers (subjects with contingent control over morphine injections) were given access to morphine (1mg/kg/injection) on a Fixed Ratio 4 (FR4) schedule of reinforcement in which they had to press the lever 4 times to receive one injection of morphine. A light located behind the opaque plastic lever manipulandum signaled drug availability. Once the lever was pressed four times, the lever light turned off, the house light turned on for 30 seconds, and a time-out period (lever presses had no consequence) was activated for the duration of drug delivery (15 sec injection) plus an additional 15 sec. The injection rate and volume were based on the animal's weight. Within each genotype, two yoked control animals were paired with the Yoker; one received an injection of 1.0 mg/kg morphine (Yoked-morphine) and the other received an injection of saline (Yoked-saline) each time the Yoker mouse self-injected morphine. All the stimulus conditions surrounding the injection were exactly the same for each member of the trio. Self-administration sessions ran for five days. Animals were housed in the operant chamber with free access to food and water. Drug availability was signaled by the lever light and occurred between 1900hr-0900hr.

Tissue harvesting & dissection

Each mouse was sacrificed by CO₂ asphyxiation and decapitation. Immediately after decapitation the brain was removed and the tissue areas, Ventral Striatum (VS) and Ventral Midbrain (VMB), were dissected and placed in separate tubes with RNA later (Sigma-Aldrich, St. Louis, MO, USA). For the fresh tissue dissection, we used a Plexiglas brain mold made for the mouse (David Kopf Instruments, Tujunga, CA, USA) and razor blades to slice the whole fresh brain into coronal slices appropriate for each section desired. Details pertaining to the brain region coordinates can be found in Supplemental Materials and Methods.

Data analysis

Only whole triads (a triad consists of Yoker, Yoked-morphine and Yoked-saline animals) that successfully completed the entire experimental protocol were considered for analysis. Thus, each Yoker subject was fully matched with their corresponding yoked controls. One group of B6 and D2 animals (N=12 per genotype, thus n=4 complete triads for each genotype) was utilized for behavioral phenotyping, gene expression profiling and quantitative real time RT-PCR (qRT-PCR) validation of expression profiles. A second cohort of B6 animals (n = 5 complete triads) served as an independent group to further validate our behavioral results and microarray gene expression findings; the behavioral results of the entire group was analyzed as described before and a subset of this group (n=3 triads) were used for qRT-PCR confirmation. The statistical comparisons between genotypes and graphical representations of the data were conducted on the first cohort, since these data represent a direct statistical association between global mRNA expression and behavior.

Water-acquisition training data analysis details can be found in Supplemental Materials and Methods.

Morphine self-administration in the first cohort of animals used for mRNA profiling was analyzed using the same statistical comparisons and procedures. The transition from initial drug exposure to a regular drug self-administration habit was characterized by the number of daily injections as well as the coefficient of variation for the inter-injection interval. An irregular drug self-administration pattern is described by a high inter-injection interval coefficient of variability while a regular drug self-administration habit is characterized by a significant decrease in the variance of the inter-injection interval as determined by a decrease in the coefficient of variation (first day of access to drug was compared to the final day of access). Analysis of morphine self-administration in the second cohort of animals used for qRT-PCR validation was conducted using a two-way repeated measures ANOVA with day as a repeated measure and yoked condition as a main effect.

Microarray Gene Expression Profiling

Microarray experimental design—Details pertaining to our custom printed cDNA microarray, microarray fabrication, experimental design, RNA isolation and labeling, hybridization protocol, quality control and quantitative real-time RT-PCR validation of microarray results can be found in Supplemental Materials and Methods, and our previous publications (Letwin et al., 2006; Tapocik et al., 2009).

Statistical and higher order microarray analysis—We utilized a hierarchical approach that mirrors the behavioral design (Reiner-Benaïm et al., 2007). Rather than testing all end hypotheses, we screened and selected hypotheses from a larger pool at the first stage based on ANOVA testing at each gene, and continued testing the selected ones using test statistics that were independent of those used from screening. In this case we constructed a hierarchical scheme of testing in which each layer of the hierarch represents one research question; testing a family of hypotheses occurs only if another (parent) hypothesis is rejected at the screening stage.

The flowchart in Figure 1 outlines the stages of analysis. Our first stage focused on the genes that were significantly altered within each genotype across Yoked condition as defined by a one-way ANOVA (per gene). Thus, at this stage of the analysis the pattern of gene expression across yoked conditions was not important. The multiplicity issue that stems from testing many genes was addressed by controlling the False Discovery Rate (FDR). In this regard we utilized the Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995) at a 10% FDR (Benjamini et al., 2001). We refer to these genes in the B6

and D2 triads as *Yoked Condition Genes (YC genes)*. In Stage 2, we used the post-hoc tests Figure of Merit (FOM) and k-means clustering (Yeung et al., 2001) to identify the dominant gene expression profiles/clusters contained in the YC dataset from B6 triads. These central expression profiles are hypothesized to represent the major gene expression circuits or networks in the self-administering B6 animals. In the third and final stage of our analysis, template matching (Pavlidis and Noble, 2001) was used to identify genes that exhibited identical expression patterns as those obtained in the dominant B6 profiles obtained in Stage 2 and YC dataset from D2 triads (e.g. gene X with the same expression pattern in both B6 and D2 triads). These overlapping genes obtained in the D2 mice would presumably represent spurious matches in the B6 triad unrelated to self-administration behavior. In other words, overlapping genes are not likely to be involved in the reinforcing properties of morphine since D2 mice did not self-administer morphine in a manner that suggests morphine served as a positive reinforcer and consequently would be omitted from higher-order bioinformatics analysis (see below). A $p < 0.05$ criteria was used to define the significance of the template matches.

A parallel series of analysis was conducted to provide specific insights into our overall findings. Pairwise gene expression comparisons (t-test with a 10% FDR) were also conducted within VS and VMB regions between the following groups: B6 Yoker vs. B6 Yoked- morphine, B6 Yoker vs. B6 Yoked-saline, D2 Yoker vs. D2 Yoked-morphine, D2 Yoker vs. D2 Yoked-saline, B6 Yoker vs. D2 Yoker, B6 Yoked-morphine vs D2 Yoked-morphine, and B6 Yoked-saline vs. D2 Yoked-saline. The comparisons were initiated for reasons described in the Results section. ANOVA, pairwise t-tests and FDR were implemented in the software program ARRAYSTAT (Imaging Research Inc., St. Catharines, ON, Canada).

Higher-order bioinformatics analysis

A series of bioinformatics steps were implemented to further explore candidate genes. These analyses (e.g. promoter, network and canonical pathway analyses) enabled our gene expression findings to be placed in a biological context for the purpose of proposing testable follow-up hypotheses. Details pertaining to higher-order analyses can be found in Supplemental Materials and Methods, and our previous publications (Letwin et al., 2006; Tapocik et al., 2009).

Results

Behavioral testing: water reinforcement training & self-administration

Water reinforcement training—All yoked conditions and genotypes were successfully trained to stable rates of lever pressing for water reinforcement. There was no overall main effect of genotype or future yoked condition in the three way repeated measures ANOVA. The number of lever presses increased each day in response to increasing ratio demands [Training Day: $F_{3,16} = 18.22$; $p < 0.0001$]. There was no significant difference in lever pressing and number of reinforcements earned among yoked conditions within either the B6 or D2 triads, and no significant difference between the yoker groups that eventually controlled drug self-administration, the B6 and D2 Yokers (Supplemental Figure 1, top and bottom panels).

Morphine Yoked Self-Administration—There was a significant main effect of genotype, yoked condition and a significant genotype X yoked condition interaction in the number of lever presses emitted [Genotype: $F_{1,18} = 7.1$; $p < 0.0158$; Yoked condition: $F_{2,18} = 10.9$; $p < 0.0008$; Genotype X Yoked condition: $F_{2,18} = 9.71$; $p < 0.0014$]. In the B6 genotype, lever pressing (and drug intake) was significantly influenced by yoked condition [Yoked

condition: $F_{2,9} = 27.3$; $p < 0.0002$] (Figure 2, **top panel**). Importantly, the Yoker group lever pressed at significantly higher rates than either Yoked control conditions on each day ($p < 0.05$). On the other hand, lever pressing in the D2 mice did not differ as a function of experimental condition [Yoked condition: $F_{2,9} = 0.12$; ns] (Figure 2, **bottom panel**). Figure 3 shows the average amount of lever pressing per day as a function of yoked condition and genotype. Lever pressing in the B6 Yoker condition exceeded all other conditions. In particular, lever pressing (and drug intake) in the B6 Yoker subjects was significantly greater than in the D2 Yoker mice [genotype: $F_{1,6} = 33.1$; $p < 0.0012$] (Figure 3). There was no trend across days in drug-taking behavior and there was no difference between yoked conditions. Overall, these results demonstrate clear morphine reinforced behavior in the B6 Yoker animal compared to the B6 Yoked controls, and no indication of morphine reinforced behavior in the D2 genotype.

B6 Yoker mice transitioned from an irregular drug self-administration pattern on the first day of access to morphine to a regular drug-taking pattern on the last day of access. The average inter-injection interval on day one was 52 min with a coefficient of variation of 0.56. In contrast, the average inter-injection interval on day five was 28 min with a coefficient of variation of 0.3. The variation in inter-injection interval decreased by nearly 50%. Data were unavailable due to technical problems for one mouse. Cumulative records of the B6 Yoker vs the B6 Yoked animals demonstrate a classical response pattern associated with positive reinforcing effects of morphine (Supplemental Figure 2). The B6 Yoker displayed intentional goal-directed lever pressing as determined by rapid run rates (time elapsed from first to fourth lever press within the Fixed Ratio). In contrast, the run rates in the B6 Yoked morphine and saline animals were significantly slower and did not show an intentional pattern to lever-pressing. The coefficient of variation for inter-injection interval was not calculated in D2 mice due to the extremely low number of injections taken.

Qualitatively similar results were found in the B6 subjects independently set aside for qRT-PCR and behavioral validation. Lever pressing (and drug intake) was significantly influenced by yoked condition [Yoked condition: $F_{2,12} = 3.94$; $p < 0.05$]. Again, the Yoker group lever pressed at significantly higher rates than either Yoked condition ($p < 0.02$ each).

Microarray gene expression analysis

ANOVA to identify YC genes—The expression profiles of ~21,000 unique genes were measured in each of the two brain regions (VS and VMB) across the Yoked triad of the B6 and D2 genotypes. In the case of the B6 genotype, a total of 1757 YC genes in the VS and 1274 YC genes in the VMB were identified, exhibiting significant (10% FDR) condition-dependent differences in expression of ~1.4-fold or greater (Figure 4A; Supplemental Table 1). This particular expression change cut-off was chosen based on empirical observations from our previous studies demonstrating that optimal success for the validation of microarray results by qRT-PCR could be achieved at 1.4-fold (Yang et al., 2002; Lee and Saeed, 2007; Liang et al., 2008; Tapocik et al., 2009). For the D2 genotype, a total of 877 YC genes in the VS and 682 YC genes in the VMB were identified at a 10% FDR with at least 1.5-fold or greater condition-dependent differences in expression (Figure 4B; Supplemental Table 1). These findings represent the genes whose expression was significantly altered by the self-administration condition, without regard to the direction of the differences between groups.

Post-hoc testing to identify 'Self-Administration' genes—In a stepwise manner we were able to converge on a set of YC genes specifically associated with morphine-reinforced behavior by: i) statistically identifying all genes altered by morphine self-administration

with regard to yoked condition, ii) refining the resolution by categorizing those gene discoveries by brain region, and iii) categorizing the genes by genotype.

FOM and k-means clustering was employed to identify the dominant expression profiles (across yoked conditions) in the *YC* gene set for the VS in B6 triads (refer to Supplemental Figure 3, panels A-H). This process identified eight main profiles (clusters A-H), and it should be noted that clusters beyond eight did not substantially improve the adjusted FOM score (Yeung et al., 2001). Four of the clusters (A, C-E) were eliminated from further consideration as a post-hoc t-test with 10% FDR indicated that the majority of genes in the Yokers were not significantly different from either the Yoked-morphine or Yoked-saline animals. These expression patterns would not be indicative of the endpoint we are specifically interested in -contingent self-administration behavior. Of the remaining clusters (B, F-H), B and H were remarkable as they contained genes that appeared to be negatively and positively correlated respectively with the behavioral profile of the B6 triad (compare Supplemental Figure 3B and H to Figure 3). In order to further refine our analysis, we subsequently used the B6 behavioral profile as a template to identify genes in clusters B and H having a significant match to the template (template match p-value < 0.05) (Figure 5). A total of 237 genes in the VS (115 positively and 122 negatively correlated genes) were identified in the B6 triad, and herein referred to as '*Self-administration*' VS genes (237 *SA* VS genes) (Figures 4A & 5; Supplemental Table 2). Using the same post-hoc testing scheme in the B6 VMB, a total of 131 genes in the VMB (117 positively and 14 negatively correlated genes) were identified (for a total of 131 *SA* VMB genes) (Figures 4A & 6; Supplemental Table 2).

In the D2 triad, a total of 21 genes in the VS (12 positively and 9 negatively correlated genes) and 86 (61 positively and 25 negatively correlated genes) in the VMB was identified by template matching (p-value < 0.05) that correlated with the behavioral profile of the B6 animals in Figure 3. We collectively refer to this set of 107 genes as '*D2-filter*' genes (Figure 4B). The *D2-filter* gene set, serving as a behavior genetics filter, was evaluated for its ability to filter out spurious template matched *SA* VS and VMB genes in the B6 triad. A total of 10 *D2-filter* genes were found in common with the *SA* gene set- these genes were removed from consideration in the *SA* gene set. Consequently, a total of 358 filtered *SA* genes have been identified upon removal of the 10 spurious gene matches as these matches are presumed not to be causally associated with drug-seeking behavior since D2 mice do not show morphine-reinforced behavior under these conditions (Figures 5 & 6). Given the low number of overlapping matches in the *SA* and *D2-filter* gene sets, the *D2* genotype filter appeared not to be as valuable in filtering out irrelevant *SA* genes as originally anticipated. This could be due to the fact that the behavioral filter (yoked condition) was much more effective than anticipated and/or the fact that D2 mice have a clearly distinct genomic response to morphine administration (supporting the original hypothesis regarding vulnerability). Notwithstanding, the *D2* genotype provides an additional assurance that the newly identified filtered *SA* genes will be functionally relevant.

Table 1 contains the T-test results (at 10% FDR) that were conducted to provide additional insight into the genomic response to self- and experimenter- administered drug. There were a significant number of VS gene expression differences in the B6 Yoker versus D2 Yoker (2540) as well as the B6 Yoked-morphine versus D2 Yoked-morphine groups (1371). Overall expression differences were anticipated in these pair-wise comparisons of genotypes given the known pharmacological responses attributed to morphine; however, the number of the differences in the Yoker comparison (2540) was much more than expected compared to the Yoked-morphine comparison (1371). Analogous pair-wise assessments of genotypes in VMB likewise revealed a greater than expected gene expression difference of 1601 in the Yoker comparison group compared to 732 in the Yoked-morphine comparison group (Table

1). Even more striking were the within genotype pair-wise assessments of B6 Yoker versus B6 Yoked-saline and B6 Yoked-morphine versus B6 Yoked-saline (Table 1 & Figure 7). Gene expression differences in the former assessment involving the contingent drug self-administering animal (Yoker) was ~5-fold greater than the latter assessment involving the passive morphine injected animal (Yoked-morphine) (1177/244 genes in VS and 879/190 genes in VMB; see Table 1 and Supplemental Table 3). Taken together, these results support the fact that there are clear genetic differences in gene expression following chronic morphine administration. Most importantly, the pairwise comparison clearly emphasizes the impact that self- versus experimenter-administered drug can have a dramatic impact on gene expression, thus making comparisons across different modes of administration difficult at best.

Identification of canonical pathways & gene networks—To address whether the genes of interest to the addiction phenotype, the SA genes shown to be selectively altered in the self-administration, reside in canonical pathways and potentially interlinked into a gene regulatory network, all filtered SA genes were computationally analyzed using microarray bioinformatics resources available through IPA, Pathway Studio, the GO Consortium, GenMAPP and KEGG. The top 10 canonical pathways and biological functions of the filtered SA genes per genotype and brain region are provided in Supplemental Table 4. The canonical pathways and biological functions reveal a strong presence of cell differentiation (i.e. cell cycle, nervous system development and function, cellular assembly and organization) cell-to-cell signaling (i.e. protein trafficking, VEGF signaling, ephrin receptor signaling) and immune response and oxidative stress signaling (cancer, glutathione metabolism, lymphocyte signaling).

The top 3 gene networks for each brain region are provided in Supplemental Table 5. The ‘self-administration’ networks may interact in such a way as to facilitate drug-seeking behavior. Figure 8 depicts an example of a ‘self-administration’ network derived from the filtered SA gene list. The left panel shows 26 of 36 genes either positively [red] or negatively [green] associated in the VS with contingent drug self-administration. Cell-to-cell signaling interaction, nervous system development and function, and cell morphology were the top functions of this network. The right panel displays 11 of 25 genes either positively or negatively associated in the VMB with contingent drug self-administration. Cell-to-cell signaling, cell death, and cellular compromise were the top three molecular functions of this network. In addition, “neuroplasticity” is exemplified as an overrepresented theme in this network ((18 out of the 37 genes) portending an association of drug-seeking behavior with dendritic and axonal neurite alteration (Figure 8). This theme is noteworthy given that repeated morphine treatment is known to alter neurofilament and other cytoskeletal proteins in the VTA, decrease the size of dopaminergic cell bodies in VTA, and inhibit neurogenesis in dentate granule cell layer (Skclair-Tavron et al., 1996; Arguello et al., 2008). A gene of considerable interest in the VS network is *H19*. This gene contains miRNA miR-675 which has been postulated to regulate dopaminergic neuron function (Freed et al., 2008) thus indicating a role of miRNAs in drug seeking behavior. The identification of *H19* as an important filtered SA gene is particularly intriguing given our recent finding that *H19* is negatively correlated with morphine tolerance (Tapocik et al., 2009).

qRT-PCR confirmation of microarray results—Six filtered SA genes (miRNA containing gene (*Mirg*), synapsin II (*Syn2*), *H19*, glutathione-s-transferase pi-1 (*Gstp1*), ADAM metalopeptidase domain 17 (*Adam17*) and tropomodulin 2 (*Tmod2*)) in the VS, and one filtered SA gene (spinophilin) in the VMB were selected for qRT-PCR confirmation. qRT-PCR confirmation was performed in the original set of RNA samples interrogated by microarray hybridizations (representing 4 complete triads, n = 4 per condition). A second set of qRT-PCR analysis was conducted in an independent set of RNA samples (n = 3 complete

triads) as a means of independent validation. Both sets of qRT-PCR samples (see genes below) confirmed the original findings. The results of the two sets were statistically compared and found not to differ from each other so they were combined in the figures (Figures 5 and 6). The correlation values of the selected genes based upon template matching (Figures 5 and 6) are provided in Supplemental Table 2.

Confirmation of miRNA's involvement was interrogated further based upon the following observations; first, as mentioned, we identified precursor miRNAs to be uniquely associated with active drug self-administration in our microarray analysis (*H19* and *Mirg*). Second, the miRNAs that are derived from these precursors play a provocative role in dopamine neuron differentiation (*H19*) and μ -opiate receptor regulation (*Mirg*). Third, the number of mRNAs thought to mediate plasticity found in our data set and the expanding evidence for a significant role for miRNAs in sculpting dendritic architecture suggests a possible link. Fourth, the network-like effects of miRNA's may help to explain the coordinated change in gene expression following chronic drug self-administration. Thus, we extended our qRT-PCR analysis to include the mature miRNAs miR-154 and miR-675, derived from the processing of transcripts *Mirg* and *H19*, respectively (Seitz et al., 2003; Cai and Cullen, 2007). In each case, qRT-PCR measurements of the 7 filtered *SA* genes in the B6 triads matched the direction and magnitude of changes measured by expression profiling with microarrays (Figure 9). Moreover, miR-154 and miR-675 qRT-PCR results mirrored expression profiles of parent mRNAs (Figure 9), suggesting successful processing of precursor transcripts to mature miRNAs by the enzyme Dicer.

Discussion

Our goal was to identify genes and gene-networks associated with the development of stable contingent morphine self-administration by utilizing a behavior-genetics strategy. The B6 and D2 animals showed significant differences in their propensity to self-administer morphine; B6 mice exhibited clear morphine reinforced behavior that was absent in the D2 genotype. These findings are in agreement with a recent report utilizing a progressive ratio schedule of i.v. morphine self-administration and morphine potentiation of rewarding brain stimulation (Elmer et al., 2010). B6 mice with contingent extended access to the drug responded at significantly greater rates than yoked controls and demonstrate an escalation in drug intake and transition from initially variable inter-injection intervals to a steady drug-taking pattern across days (see Vendruscolo et al., 2011). Furthermore, we identified 358 unique genes (filtered *SA* genes) in the VS and VMB that were either positively or negatively associated with contingent morphine reinforced behavior. Gene expression and bioinformatics analysis of this dataset identified overrepresented themes of neuroplasticity, axonal guidance, and the miRNA pathway as being involved in morphine-reinforced behavior.

The behavioral and molecular consequences of self- versus non-contingent drug administration were robust. The differences between conditions involve varied neurosystems and are likely linked to interactions with motivational state sets and conditioned compensatory responses (Weise-Kelly and Siegel, 2001; Kiyatkin and Wise, 2002; Kuntz et al., 2008; LaLumiere and Kalivas, 2008; Weber et al., 2009). We utilized the yoked-control paradigm in order to eliminate from consideration any genes merely altered by the surgical (yoked-saline) and experimental procedures (yoked-morphine) and those unrelated to intentional drug-taking behavior (yoker vs yoked-morphine and yoked-saline). In a strict genetic comparison of only the animals self-administering the drug (Yoker B6 vs Yoker D2) one would have been left with a large number of candidate genes to consider (Table 1: 2540 in VS, 1601 in VMB). On the other hand, roughly half that number was found to differ between genotypes injected with morphine but without control over the injections (yoked-

morphine comparisons). The interesting dynamics of self- versus non-contingent administration is further revealed by contrasting active versus passive administration within genotype, especially within the VS. In the B6 genotype that actively seeks the reinforcing properties of morphine, there was a five-fold greater number of genes with altered expression levels in the active (1177 genes) versus passive (244 genes) administration groups compared to the yoked-saline condition. In stark contrast, in the genotype for which morphine was not reinforcing, D2, the number of genes whose expression level was altered by morphine administration was significantly less (107 versus 262). There is a limited chance that a subset of candidate genes identified in the SA set are not uniquely associated with morphine-reinforced behavior but may be related to the learned instrumental response (lever pressing) to obtain generic reward. A yoked control group responding for a natural reward at matched reinforcement magnitude and frequency could further refine the SA gene list in future studies. Overall, implementing the genome-wide screen using full template-matching and genotype filter procedures revealed genes uniquely altered by active drug self-administration and significantly narrowed the focus of high value candidate genes.

Morphine is one of the most efficacious drugs available for the treatment of pain, however chronic administration can have devastating consequences, namely tolerance and addiction, in a subset of the population. Understanding the genes and molecular mechanisms that lead to tolerance and addiction could potentially alleviate these consequences. Only a small number of genes (17) were found to overlap between our filtered SA (present study) and tolerance genes (Tapocik et al., 2009). This was not surprising given the fact that our tolerance paradigm was specifically designed to identify genes involved in passive intermittent morphine administration that leads to analgesic tolerance (using a behavior genetics filter to focus on phenotype-specific associations) whereas the self-administration experimental model was specifically designed to discover genes involved in active morphine *self*-administration. Even though there are valid reasons for the small overlap, a few particularly interesting genes did overlap and deserve further investigation. Spinophilin was found positively correlated with the development of tolerance (Tapocik et al., 2009) and active self administration in the TL and VMB, respectively. Spinophilin is physically associated with the mu-opioid receptor and modulates mu-opioid receptor signaling and endocytosis (Charlton et al., 2008). Spinophilin is a dendritic spine-enriched scaffold protein that is known to modulate synaptic transmission as well as dopamine mediated plasticity in the striatum and interacts with RGS (Regulator of G-protein signaling) proteins (Allen et al., 2006). Additionally, spinophilin is also known to bind to PP1, a serine/threonine phosphatase that is intimately involved in the regulation of synaptic strength at excitatory synapses (Allen et al., 1997). Therefore, it is likely that spinophilin plays a critical role in mu-opioid receptor signal transduction and desensitization and, consequently, morphine tolerance and addiction. Spinophilin could serve as a pharmacological target to prevent the development of tolerance and reduce addiction liability.

There was very little overlap between the filtered SA genes identified in this study and other studies comparing inbred strains or self administration in rats - this is not surprising since the current study was uniquely designed to identify only those genes involved in active morphine self- administration. No genes identified in this study overlap with an earlier subtractive hybridization study using a heroin yoked-control paradigm (25 genes in NAc; (Jacobs et al., 2002, 2004)). However, species (rat vs. mouse), drug (heroin vs. morphine), stimulus presentation (cue presented prior to drug delivery in Yoked-heroin group) and time of tissue harvest (3 week abstinent vs 6 hrs) make a direct comparison difficult. In other studies conducted in inbred mouse strains, experimenter administered morphine results in a very large number of changes in gene expression (Loguinov et al., 2001(medial striatum); Ammon et al., 2003(frontal cortex); Rodriguez-Parkitna et al., 2004 (amygdala)). Gene expression profiling in four inbred strains (B6, 129P3/J, SWR/J, D2) (Korostynski et al.,

2007) and B6 and D2 mice (Grice et al., 2007) identified experimenter-delivered morphine-induced gene expression differences in the striatum and nucleus accumbens, respectively. Only three genes were found to overlap between our studies and the Korostynksi et al., study (growth arrest specific 5 (*Gas5*), xanthine dehydrogenase (*Xdh*), and *inaD*-like *Drosophila* (*Inadh*)) while no genes overlapped with the Grice et al., study. We propose that the small overlap in genes is likely due to the differences in morphine dose, brain region, and self- vs. experimenter drug administration.

Bioinformatics strategies can place candidate genes in canonical pathways and gene networks in order to highlight their functional relevance (Letwin et al., 2006; Tapocik et al., 2009). Canonical pathway analysis and gene network analysis of the filtered *SA* genes strongly implicate a significant role for neuroadaptation, axonal and dendritic development and neurite outgrowth. Alterations in the expression of these *SA* genes may increase or decrease neuron connections through pruning or neurite outgrowth. The *SA* genes displayed in the neurite outgrowth network in Figure 8 have been shown individually to be involved in neuritogenesis and some form of neuroadaptation process. Moreover, many of the genes in the neurite outgrowth network (Figure 8), including Kalirin (*Kalrn*) (Penzes and Jones, 2008), *Rims1* (Lonart, 2002; Schoch et al., 2002), synapsin II (*Syn2*) (Rosahl et al., 1995), and tropomodulin 2 (*Tmod2*) (Cox et al., 2003), have been shown to be involved in neurotransmitter release, LTP and LTD, as well as synaptic plasticity in the adult brain. Previous studies provide strong support for the current findings of a specific role for altered dendritic morphology in response to chronic opioid administration (Robinson and Kolb, 1999; Liao et al., 2005; Liao et al., 2007) including evidence that contingency plays a role (Robinson et al., 2002). The changes described above may alter how neurons integrate excitatory and inhibitory neurotransmission (see (Dumont et al., 2008)). Thus, gene expression influencing the reorganization of synapses suggests that some of the long-term effects of morphine may be due to its ability to remodel dendrites and axonal projections. While the underlying assumption that the changes in mRNA expression correspond to changes in protein levels (rather than a compensatory response) clearly needs confirmation (see Mukherji et al., 2011), the discovery of these particular gene networks and canonical pathways remains intriguing and warrants further investigation.

The potential role of miRNAs in response to drug self-administration was a particularly interesting finding in this study especially as it relates to the canonical pathways and gene networks described above. miRNAs are prime candidates to mediate network-like changes in responses to chronic drug administration since they have been shown to play a role in sculpting dendritic architecture in an activity-dependent manner (Kosik, 2006; Schratt et al., 2006; Wayman et al., 2008). Of particular interest, two primary miRNA genes (*H19* and *Mirg*) were empirically found to be significantly associated with morphine self-administration. *Mirg* (miRNA containing gene) was found to be significantly associated with morphine reinforced behavior in the VS. A search of the miRBase Targets database (www.mirbase.org) for predicted target mRNAs of miR-154 (mature miRNA derived from *Mirg*, (Seitz et al., 2003)) identified 4 *SA* genes (*Fxyd4*, *Grm3*, *Odf2l* and *Slc4a4*) as potential targets. *Grm3* or metabotropic glutamate receptor 3 has been critically associated with LTP, LTD and synaptic plasticity in the hippocampus (Poschel et al., 2005). Perhaps even more interesting was the finding that the mu-opioid receptor mRNA also serves as a significant candidate target of miR-154 ($P=0.017$), indicating a potential role of miRNAs in regulating the mu-opioid receptor function. These results, along with recent findings related to miR-23b (Wu et al., 2008; Wu et al., 2009) indicate another level of regulation for the mu-opioid receptor. *H19* is intriguing since it was found negatively associated with our self-administration (present study) and tolerance data (Tapocik et al., 2009) in the VS and PAG, respectively. This primary miRNA gene gives rise to miR-675 in humans and mice (Cai and Cullen, 2007) and the *H19* imprinting region is involved in dopamine neuron differentiation

(Freed et al., 2008). Since *H19* was found associated with tolerance and self-administration it may be advantageous to pharmacologically target this candidate in chronic drug administration settings. The significant amount of support for neuroplastic changes involved in drug abuse (Kalivas and O'Brien, 2008; Kauer and Melenka, 2007; McClung and Nestler, 2008) and the proposed canonical pathways and biological functions of axonal guidance, cytoskeletal signaling and cancer along with the implication of miRNA involvement suggest that the nervous system may engage a response network designed to mediate the chronic insult of extended drug self-administration. When the direction of change for each candidate gene in Figure 8 is taken into consideration, the results suggest a dynamic interplay between processes actively employed to increase synaptic plasticity, connections and differentiation-like response (VMB: DOK1, SHC1, MMP2, CHRN2, IL11RA; VS: ADAM17, GRM3, LCN2, PTTG1, VTN, CRK, KALRN, SPARC, TMOD2, GSTP1) versus those that would result in a diminished engagement of the aforementioned processes (VMB: ACTB, TNFRSF19, MAST2; VS: ADCYAP1R1, FZD3, H19, RIMS1, SRGAP3, SYN2, SYT1). The prominent role for miRNAs in cancer, transdifferentiation and metaplasia (Zhang et al., 2007; Corsten et al., 2007; Papagiannakopoulos and Kosik, 2008) and our identified candidate mRNAs reputed to play a role in these processes (promote: DOK1, SHC1, MMP2, LGALS7, PTTG1, VTN; diminished effect: H19, TNFRSF19) suggest that the overall net response to chronic drug exposure may represent a variant of transdifferentiation (Slack and Tosh, 2001; Scott et al., 2005; Ayesh et al., 2002).

We postulate that the coordinated regulation of our neuroplasticity gene network represents a C57-specific 'regulon' involved in self-administration behavior (Figure 8). In order to identify potential molecular underpinnings controlling this regulon, we applied two computational approaches. In the first approach, we employed a web-accessible tool that utilizes the miRanda algorithm (www.microrna.org) to identify the mRNAs in the neuroplasticity network that are targeted by miRNAs. Remarkably, two miRNAs (mmu-miR-186, mmu-miR-381) were each found to putatively target nearly a third of the mRNAs (7 out of 24) displayed in the C57 VS neuroplasticity network (Figure 8, **left panel**; only miRNA:mRNA pairings with high mirSVR scores were considered). In the second approach, we computationally searched for an over-representation of TF binding site(s) in common to the promoter regions of the genes comprising the neuroplasticity network. The presence of a common over-represented TF binding site would be indicative of co-regulation of genes within a regulon. Our analysis indicates that the AP-1 binding site is significantly over-represented ($P = 0.009154$), having been found in 14 out of 19 gene promoters (19 out of 24 network genes had at least 1 kb of promoter sequence for analysis). This finding is particularly germane as delta FosB has been shown to bind to the AP-1 site, accumulate in a subset of neurons in the nucleus accumbens and dorsal striatum, and serve as a molecular switch for drug addiction (Nestler et al., 2001). In summary, the two potential regulatory scenarios described above await future experimental validation.

In summary, our studies have identified candidate genes and gene circuitries involved in morphine reinforced behavior. Neurite outgrowth, neuroplasticity, and miRNA mechanisms in general appear to play a defining role in drug-seeking behavior. A dynamic picture is presented in which mechanisms that would promote neuronal remodeling are balanced in part by those that would either oppose or permit diminished neuronal survival or remodeling. The genes identified may ultimately provide us with potential therapeutic targets to help decrease addiction liability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Step 1: OBTAINING YOKED CONDITION GENES (YC Genes)

ANOVA: Find significant VS genes at 10% FDR that vary in the B6 and D2 triads

Step 2a: OBTAINING PREDOMINANT EXPRESSION CLUSTERS

Post-hoc: Use FOM and k-means clustering on B6 YC gene dataset

Step 2b: BIOLOGICAL CONTEXTUALIZATION

Identify B6 clusters containing genes expressed in Yoker that differs significantly from both Yoked-Morphine & Yoked-Saline

Paring down gene set by noting that 2 of 4 clusters mimic B6 triad SA behavior. Use morphine SA behavior in B6 triad as template to select positive and negative correlated VS genes in B6 triads

Correlated SA Genes from VS of B6 Triads **Step 3: FILTERING GENE SETS**

Omit template matched SA genes in B6 triads having similar expression pattern in D2 YC gene set (D2-filter genes)

Figure 1.

Flowchart outlining behavior-genetics strategy to identify candidate self-administration (SA) genes.

Figure 2.

Intravenous morphine self-administration in B6 (Top Panel) and D2 (Bottom Panel) mice as a function of yoked condition. Yoker mice had contingent control over drug injections; every fourth lever press resulted in an intravenous delivery of 1.0 mg/kg morphine. Each time that the Yoker self-administered the drug the Yoked subjects received an injection of the same dose of morphine (Yoked-morphine) or saline (Yoked-saline). Each point (both panels) represents the mean of the four animals ($\pm$ S.E.M) that were eventually used in mRNA profiling.

Figure 3.

Average number of lever presses in B6 and D2 mice as a function of Yoked condition. Each bar represents the five day average number of lever presses in each genotype. Each bar represents the mean of the four animals ($\pm$ S.E.M) that were eventually used in mRNA profiling. The B6 Yoker group lever pressed significantly more than all other experimental groups (*, $p < 0.001$).

Figure 4.
The number of significant ANOVA (black bars) and Template Match (slashed bars) genes found in the VS and VMB of the B6 (panel A) and D2 animals (panel B).

Figure 5.

Coordinately-regulated genes identified by template matching in the VS of B6 animals. Gene profiles indicated by gray lines were positively (top left graph) or negatively correlated (bottom left graph) with contingent morphine self-administration behavior in B6 animals. The two right graphs depict the expression pattern of the same genes in the VS of D2 animals. Note the mostly random pattern of D2 gene expression. Red lines represent the average profile of genes. Five D2 genes that follow the same template as the B6 animals (indicated by the blue lines in the right graphs) were removed from consideration as SA genes. Insets in each graph represent gene clustergrams where high and low expression is represented as red and green, respectively. Data presented are the mean of 4 independent animals per yoked condition.

Figure 6. Coordinately-regulated genes identified by template matching in the VMB of B6 animals. Gene profiles indicated by gray lines were positively (top left graph) or negatively correlated (bottom left graph) with contingent morphine self-administration behavior in B6 animals. The two right graphs depict the expression pattern of the same genes in the VMB of D2 animals. Note the mostly random pattern of D2 gene expression. Red lines represent the average profile of genes. Five D2 genes that follow the same template as the B6 animals (indicated by the blue lines in the right graphs) were removed from consideration as SA genes. Insets in each graph represent gene clustergrams where high and low expression is represented as red and green, respectively. Data presented are the mean of 4 independent animals per yoked condition.

Figure 7.

Venn diagram depicting differentially expressed genes derived from pair-wise assessments of B6 Yoker versus B6 Yoked-saline and B6 Yoked-morphine versus B6 Yoked-saline in VS and VMB. Operational differences in transcriptional activity are strikingly evident between contingent morphine self-administration and passive morphine administration.

Figure 8.

Neurite outgrowth gene network in the VS (left) and VMB (right) of B6 animals. The network connections were computationally constructed from the self-administration (SA) gene set (see Supplemental Table 1). Edges (lines) connecting nodes (SA genes) represent regulatory interactions such as regulation of gene expression, protein-protein interactions, protein-nucleic acid interactions, or protein-hormone interactions. Positively correlated genes are indicated in red, non-correlated in gray, and negatively correlated in green. Intensity of green or red shading in nodes indicates relative gene expression. Definitions of gene symbol abbreviations in VMB are DOK1, docking protein1; SHC1, src homology 2 domain-containing transforming protein C1; CD55, CD55 antigen; GNA13, guanine nucleotide binding protein, alpha 13; MMP2, matrix metalloproteinase 2; ACTB, actin, beta, cytoplasmic; FOSL1, fos-like antigen 1; CHRN2, cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal); IL11RA, interleukin 11 receptor, alpha chain 1; TNFRSF19, tumor necrosis factor receptor superfamily, member 19; MAST2, microtubule associated serine/threonine kinase 2. Definitions of gene symbol abbreviations in VS are AGRN, agrin; KALRN, RhoGEF kinase; NFkB, nuclear factor kappa-light-chainenhancer of activated B cells); SRGAP3, SLIT-ROBO Rho GTPase activating protein 3; SYN2, synapsin II; SNAP25, synaptosomal-associated protein 25; VTN, vitronectin; CRK, v-crk sarcoma virus CT10 oncogene homolog; SPARC, secreted acidic cysteine rich glycoprotein; LGALS7, lectin, galactose binding, soluble 7; LCN2, lipocalin 2; ADAM17, a disintegrin and metalloproteinase domain 17; FZD3, frizzled homologue 3; H19, H19 fetal liver mRNA; PTTG1, pituitary tumor-transforming 1; TMOD2, tropomodulin 2; TPM3, tropomyosin 3, gamma; ADCYAP1R1, adenylate cyclase activating polypeptide 1 receptor 1; RIMS1, regulating synaptic membrane exocytosis 1; NRCAM, neuron-glia-CAM-related cell adhesion molecule; SLC6A6, solute carrier family 6 (neurotransmitter transporter, taurine), member 6; GRM3, metabotropic glutamate receptor 3 protein; GSTP1, glutathione S-transferase, pi 1, RASGRF2, RAS protein-specific guanine nucleotide-releasing factor 2; CACNB2, calcium channel, voltage-dependent, beta 2 subunit.

Figure 9.

Quantitative real time RT-PCR validation of microarray data. Comparison of individual template matched genes measured by microarray analysis (black bars) to qRT-PCR (white bars). Genes validated in VS were H19 (H19 fetal liver mRNA), Adam17 (a disintegrin and metalloproteinase domain 17), Spinophilin, Mirg (miRNA containing gene), Tmod2 (tropomodulin 2), and Gstp1 (glutathione-s-transferase pi-1). Syn2 (synapsin II) was validated in VMB. In the bottom two panels (middle and right), miR-675 and miR-154 levels in the VS were validated by qRT-PCR. Note that levels of miR-675 and miR-154 correspond directly with expression levels of their primary miRNA transcripts H19 and Mirg, respectively. Data are represented as the mean $\pm$ S.E.M. of 4 independent animals per yoked condition for microarray experiments and 7 independent animals per yoked condition for qRT-PCR determinations. *, Significantly different from Yoker animals, $p < 0.05$. For each gene, the correlation between microarray and RT-PCR data was significant at $p < 0.05$ (gene, Pearson correlation coefficient): H19, $r = 0.998$; Adam17, $r = 0.999$; Spinophilin, $r = 0.975$; Mirg, $r = 0.993$; Syn2, $r = 0.999$; Tmod2, $r = 0.986$; Gstp1, $r = 0.994$; H19/miR-675, $r = 0.991$; Mirg/miR-154, $r = 0.990$). VS, ventral striatum; VMB, ventral midbrain.

Table 1

Number of Differentially Expressed Genes in Pairwise Comparisons

	VS	VMB
B6 Yoker vs. D2 Yoker	2540	1601
B6 Yoked-morphine vs. D2 Yoked-morphine	1371	732
B6 Yoked-saline vs. D2 Yoked-saline	842	1574
B6 Yoker vs. B6 Yoked-morphine	927	325
B6 Yoker vs. B6 Yoked-saline	1176	879
B6 Yoked-morphine vs. B6 Yoked-saline	244	190
D2 Yoker vs. D2 Yoked-morphine	256	292
D2 Yoker vs. D2 Yoked-saline	107	207
D2 Yoked-morphine vs. D2 Yoked-saline	262	109

Pairwise comparisons by t-test with 10% FDR