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Analysis of candidate genes for morphine preference quantitative trait locus *Mop2*

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Abstract

Compared to DBA/2J (D2), C57BL/6J (B6) inbred mice exhibit strong morphine preference when tested using a two-bottle choice drinking paradigm. A morphine preference quantitative trait locus (QTL), *Mop2*, was originally mapped to proximal chromosome 10 using a B6xD2 F2 intercross population, confirmed with reciprocal congenic strains and fine mapped with recombinant congenic strains. These efforts identified a ~10 Mbp interval, underlying *Mop2*, containing 35 genes. To further reduce the interval, mice from the D2.B6-*Mop2-P1* congenic strain were backcrossed to parental D2 mice and two new recombinant strains of interest were generated: D2.B6-*Mop2-P1.pD.dB* and D2.B6-*Mop2-P1.pD.dD*. Results obtained from testing these strains in the two-bottle choice drinking paradigm suggest that the gene(s) responsible for the *Mop2* QTL is one or more of 22 remaining within the newly defined interval (~7.6 Mbp) which includes *Oprm1* and several other genes related to opioid pharmacology. Real-time qRT-PCR analysis of *Oprm1* and opioid-related genes *Rgs17*, *Ppp1r14c*, *Vip*, and *Idy* revealed both between-strain and within-strain expression differences in comparisons of saline- and morphine-treated B6 and D2 mice. Analysis of *Rgs17* protein levels also revealed both between-strain and within-strain differences in comparisons of saline- and morphine-treated B6 and D2 mice. Results suggest that the *Mop2* QTL represents the combined influence of multiple genetic variants on morphine preference in these two strains. Relative contributions of each variant remain to be determined.

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Keywords

μ -opioid receptor; two-bottle choice; *Rgs17*; *Ppp1r14c*; *Iyd*; *Vip*

1.1 Introduction

For over 30 years, the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains have been studied as a model system for substance abuse, exhibiting robust differences in responses observed following voluntary morphine intake (by liquid, food, or operant intravenous self administration), with B6 mice consistently showing increased preference and willingness to work for opioids (Belknap et al., 1993; Horowitz et al., 1977; Suzuki et al., 1991; Elmer et al., 2010). In addition, B6 and D2 mice exhibit differences in the development of tolerance (Frigeni et al., 1981) and dependence, (Belknap, 1990; Suzuki et al., 1991) withdrawal symptoms (Kest et al., 2002; Suzuki et al., 1991), analgesia (anti-nociception) (Belknap et al., 1995; Bergeson et al., 2001), Straub tail (Belknap, 1990), hypothermia (Muraki and Kato, 1987) and reward as determined by conditioned place preference (Cunningham et al., 1992; Orsini et al., 2005).

The dramatic contrast between B6 and D2 mice in morphine preference behavior was first studied using a two-bottle choice paradigm (Horowitz et al., 1977) and was confirmed in a subsequent study using a slightly modified testing protocol (Belknap et al., 1993). A genetic basis for these differential responses was documented in a quantitative trait loci (QTL) mapping study in which three QTL were detected that together explained over 85% of the observed genetic variance – two major QTL on chromosomes (Chr) 10 and 6, and a suggestive QTL on Chr 1 (Berrettini et al., 1994b). Our studies with double congenic mice (Doyle et al., 2008) indicated that the Chr 6 QTL (Berrettini et al., 1994b) could be explained by strain polymorphisms at the *qui* locus which maps to Chr 6 (Blizzard et al., 1999; Nelson et al., 2005) and produces taste aversion to quinine (used in the control bottle *versus* morphine) in B6 mice (Lush, 1984). Fine-mapping with congenic strains narrowed the Chr 10 QTL critical interval to ~4.5 cM containing 35 genes (Doyle et al., 2008; Ferraro et al., 2005) including *Oprm1* which encodes the μ -opioid receptor (MOR), the main receptor for morphine in the central nervous system (Pan, 2005; Pasternak, 2001). The role of the MOR in mediating the effects of morphine, including analgesia, tolerance, dependence and withdrawal, respiratory depression and hypothermia, has been confirmed in various MOR knockout (MuKO) mouse lines (Loh et al., 1998; Matthes et al., 1996; Schuller et al., 1999; Sora et al., 1997; Tian et al., 1997). Thus, an obvious candidate gene for controlling differences in morphine consumption and preference between B6 and D2 mice located near the peak of the initial Chr 10 QTL linkage map and within the critical genomic region introgressed in congenic strains is *Oprm1*. However, in addition to *Oprm1*, the *Mop2* critical interval harbors several other genes that have been documented to be involved in the actions or effects of opioids. The present work analyzes 5 candidate genes; [proportional]-opioid receptor (*Oprm1*; GRCm38/mm10 build genomic base pair position 10:6,788,570-7,038,209), iodotyrosine deiodinase (*Iyd*; *a.k.a.* *Dehal1*, 10:3,540,279-3,554,877), vasoactive intestinal polypeptide (*Vip*, 10:5,639,218-5,647,614), regulator of G-protein signaling 17 (*Rgs17*; *a.k.a.* *RgsZ2*, 10:5,825,664-5,922,400) and

protein phosphatase 1 (PP1) regulatory (inhibitor) subunit 14c (*Ppp1r14c*; a.k.a. *KEPI*, 10:3,366,150-3,464,975), defined by the congenic strain approach, that could explain, at least in part, the morphine preference QTL (*Mop2*), as well as other morphine-based quantitative traits that map to the proximal region of mouse Chr 10 (Belknap et al, 1995; Bergeson et al, 2001).

2.1 Experimental Procedures

2.1.1 Animals

The animals used in these studies were derived from B6 and D2 mice originally purchased from the Jackson Laboratory (Bar Harbor, ME) and propagated at our animal colony at the Veterans Affairs Medical Center, Coatesville, PA. Genetic drift in the colony is minimized by supplementing breeders with additional animals purchased from the Jackson Laboratory several times per year. Mice are weaned at 3-4 weeks of age and group-housed by sex until used for experimentation. They are kept on a light/dark cycle of 14 h on (lights on at 0700 h) and 10 h off and have free access to food. Water is *ad libitum* except during the two-bottle choice paradigm described below. All studies were approved by the Institutional Animal Care and Use Committees governing the participating laboratories.

The congenic mice used in these studies (see Figure 1) were created in our laboratory with a marker-assisted breeding strategy as described (Doyle et al., 2008; Ferraro et al., 2005). The D2.B6-*Mop2-P1-pD.dB* subcongenic strain was derived by backcrossing heterozygous D2.B6-*Mop2-P1* congenic mice (Doyle et al., 2008) to parental D2 mice and tracking recombination by marker analysis within the original introgressed region. To propagate mice for the two-bottle choice experiments, the D2.B6-*Mop2-P1-pD.dB* strain was made heterozygous across the introgressed region by backcrossing to D2 mice. Heterozygous D2.B6-*Mop2-P1-pD.dB* mice were mated together to create homozygous D2.B6-*Mop2-P1-pD.dB* or D2.B6-*Mop2-P1-pD.dD* congenic mice for use in these studies. Thus, D2.B6-*Mop2-P1-pD.dD* congenic mice are the “control” littermates of crosses of the heterozygous D2.B6-*Mop2-P1-pD.dB* mice, and are essentially genetic equivalents of the D2 strain, residual components of B6 genome from congenic strain production notwithstanding.

2.1.2 Genotyping

Analysis of DNA markers *D10Mit166* and *D10Mit28* was performed by PCR amplification followed by electrophoresis of products on polyacrylamide gels as described previously (Berrettini et al., 1994b). A single nucleotide polymorphism (SNP) between B6 and D2 mice in exon 10 of *Oprm1* that creates a *DdeI* restriction site was used to genotype congenic mice at this genetic locus as described previously (Ferraro et al., 2005). Primer sequences, PCR parameters and RFLP digestion protocols for genotyping of SNPs within *Ppp1r14c* (a.k.a. *KEPI*), *Ulp1* (a.k.a. *9230019H11Rik*), *Lrp11*, *Pcmt1* and *Katna1* are available upon request.

2.1.3 Two-bottle Choice Test for Morphine Preference

Eight to ten week old male mice were housed individually for the duration of the 12-day two-bottle choice drinking test. Mice had free access to food throughout the experiment. The

morphine/quinine two-bottle choice test was performed as described previously (Doyle et al., 2008). Morphine consumption and preference scores were calculated as described previously (Berrettini et al., 1994b; Ferraro et al., 2005).

2.1.4 Gene Expression

Several genes within the introgressed critical region have a biological relationship to opioid pharmacology and were selected for expression analysis. Selection was based on PubMed literature searches that included the search terms “opioid”, “opiate” or “morphine” in combination with names or symbols of each of the 22 genes remaining within the critical interval. Brain tissue was obtained from parental B6 or D2 mice that were experimentally naïve or that were 5-days post-surgery for implantation of sham (placebo control) or morphine (25 mg) pellets. Total RNA was prepared from whole brain or specific brain regions using the Trizol® reagent method as recommended by the manufacturer (Life Technologies, Grand Island, NY USA). RNA was quantified by UV spectrophotometry. Integrity of RNA was assessed by visualization of 28S and 18S rRNA bands under ultraviolet illumination after denaturing formaldehyde gel electrophoresis in the presence of ethidium bromide. Prior to reverse transcription, equivalent amounts of total RNA (typically 2 µg) were treated with RNase-free DNase I (Sigma-Aldrich, Saint Louis, MO USA) at 25°C for 15 minutes and EDTA stop solution added to chelate Mg²⁺ ions before heat-inactivation of DNase I at 70°C for 10 minutes. For whole brain samples, total RNA was reverse transcribed into cDNA with random hexamers for gene expression assays. For discrete brain region gene expression analysis, the polyadenylated mRNA portion of total RNA was reverse transcribed with oligomer(dT)₁₈ using the Superscript II® first strand cDNA synthesis kit under conditions recommended by the manufacturer (Life Technologies). Steady-state mRNA levels were determined in triplicate by real-time qRT-PCR in a 7900HT instrument using the following TaqMan® gene expression assays: *Oprm1* exon 2-3 (Mm01188089_m1), *Oprm1* exon 3-4 (Mm00565144_m1), *Oprm1* custom-designed MOR-1 long 3'UTR (Mm_Mu3'UTR), *Rgs17* (Mm00490876_m1), *Ppp1r14c* (Mm00652462_m1), *Id1* (Mm00503564_m1), *Vip* (Mm00660234_m1) and *Actb* endogenous control (4352341E; used to normalize). With the exception of the custom-designed *Oprm1* MOR-1 long 3'UTR assay, all assays were designed to cross an exon-exon junction and were determined to have a PCR efficiency within 10% of 100% established by the manufacturer. Replicate determinations for each assay were done in 384 well plates in a total reaction volume of 20 µl which was composed of 10 µl 2X TaqMan® gene expression master mix, 1 µl 20X gene expression assay mix, 4 µl cDNA (~1 ng converted mRNA input) and 5 µl water. Reactions were cycled under standard PCR conditions and fluorescence readings taken in real-time on a 384-well block-equipped 7900HT as recommended by the manufacturer (Life Technologies). The baseline and threshold for each assay were determined automatically with automatic removal of technical replicate outliers in a relative quantification study using the SDS 2.2.2 software (Life Technologies). Replicate cycle thresholds (C_T) were averaged for the target and endogenous control genes and C_T determined for each biological sample. The C_T values were averaged prior to calculation of the C_T for use in fold-difference determination by the 2^{-(C_T)} method (Livak and Schmittgen, 2001). Lower and upper ranges were calculated using the formula 2^{-(C_T±SD)}

and $2^{-(\text{Ct}-\text{SD})}$, respectively, where SD is the standard deviation of the Ct determination.

2.1.5 Rgs17 Protein Expression analysis

Brain tissue was obtained from parental B6 or D2 mice that were 5-days post-surgery for implantation of sham (placebo control) or morphine (25 mg) pellets (N=3 for each group). Brain regions were dissected and homogenized in ice cold protein extraction buffer (100 mM Tris-HCl; pH 7.4, 150mM NaCl, 15% glycerol, 2% Triton X-100, 1 mM DTT, 0.5 mM PMSF and 1X Complete mini protease inhibitors (Roche)), and the extract spun at 21,000g for 10 min at 4°C to pellet nuclei, mitochondria and insoluble proteins. The supernatant containing the soluble proteins was taken as brain region extracts. Total protein concentrations for each extract were determined by the bichinchonic acid (BCA) protein assay (Thermo-Scientific, Rockford, IL, USA). For immunoblot analysis, 50 µg of total protein extract from each brain region was separated on a 4-12% Bis-Tris NuPage gel in 1X MOPS buffer. The gels were transferred to nitrocellulose using an iBlot (Thermo-Scientific, Rockford, IL, USA) semi-dry blotter, blots were stained with Ponceau S and then blocked in 10% non-fat dry milk in 1X Tris-buffered saline containing 0.1% Tween-20 (TBST). Blots were incubated with a 1:5000 dilution of mouse anti-[.beta]-actin antibody (MAB1501, Millipore, Temecula, CA, USA) for 1 hr at room temperature in TBST containing 5% non-fat dry milk. Blots were incubated overnight at 4°C in 1X Odyssey blocking reagent (Li-Cor, Lincoln, NE, USA) containing a 1:1000 dilution of chicken anti-Rgs17 (GTx14260, GeneTex, Irvine, CA, USA). Blots were then washed in 1X phosphate-buffered saline containing 0.1% Tween-20 (PBST), then incubated in 1:10,000 dilution of donkey anti-chicken IRDye-680LT (Li-Cor) and donkey anti-mouse IRDye-800RW (Li-Cor) secondary antibodies in 1X Odyssey block reagent at room temperature for 1 hr. Blots were washed in PBST and then rinsed in PBS before scanning on an Odyssey infra-red scanner at settings of 169 micron resolution, quality set to high, 700 nm intensity of 5 and 800 nm intensity of 7. Multi-color images were exported in .tif format in separate grayscale images. 700 nm grayscale (Rgs17) and 800 nm grayscale (β-actin) bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) software. Band intensities were measured and the ratio of Rgs17/β-actin taken for each lane. The ratio averages and standard deviations were taken for each group (N=3) and the results graphed using Excel 2007 (Microsoft, Redmond, WA, USA).

2.1.6 Statistical analysis

Graphs were made using either Microsoft Office Excel 2007 (Redmond, WA, USA) or GraphPad Prism software version 4.0 (San Diego, CA USA). Statistical analyses were performed using JMP 8.0 (SAS Institute, Inc., Cary, NC USA) or GraphPad Prism 4.0 software. Two-bottle choice drinking data were compared by ANOVA with Tukey-Kramer Honestly Significant Difference (HSD) *post-hoc* analysis with alpha set to 0.05. Grubb's test (<http://www.graphpad.com/quickcalcs/grubbs1/>) was used to determine Ct value outliers among biological replicates prior to final analysis by Wilcoxon rank sum test. The Wilcoxon rank sum test was used to determine statistical significance of gene expression data by comparisons of the average replicate Ct values (Gene - β-actin) between strains (sham- *versus* sham-pellet implanted) or within each strain (sham- *versus* morphine-pellet).

implanted). Rgs17/ β -actin protein ratio averages were compared by one-way ANOVA and statistical significance ($p < 0.05$) was determined by non-parametric Wilcoxon rank sum test.

3.1 Results

3.1.1 Two-bottle choice experiments

Since the time of the initial report on the presence of a QTL on proximal Chr 10 for voluntary morphine consumption and preference (Berrettini et al., 1994b), we have continued to backcross the D2.B6-*Mop2* mice (Ferraro et al., 2005) to their background parental strain (D2) to generate subcongenic lines for refined QTL mapping (Doyle et al., 2008). Thus, we created the D2.B6-*Mop2-P1* and D2.B6-*Mop2-D1* subcongenic strains. Further backcrossing of the D2.B6-*Mop2-P1* subcongenic strain recently led to a new Chr 10 recombinant, namely D2.B6-*P1.pD.dB* and its littermate control, namely D2.B6-*P1.pD.dD* (see Figure 1 for map).

We tested these new congenic strains (Figure 1, strains D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD*) for morphine consumption and preference in a two-bottle choice paradigm as described (Berrettini et al., 1994b; Ferraro et al., 2005; Doyle et al., 2008). Figures 2 and 3 show the results of these experiments. Levels of morphine consumption were significantly different among strains (ANOVA with Tukey-Kramer HSD post-hoc: $F(6,49)=33.82$; $p < 0.0001$) with B6 different from all other strains ($p < 0.0001$), D2.B6-*Mop2* and D2.B6-*Mop2-P1* statistically equivalent to each other ($p=0.999$), but different from D2, D2.B6-*Mop2-D1*, D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD* ($p < 0.05$ to $p < 0.01$; Figure 2). Levels of quinine consumption were statistically different among strains (ANOVA with Tukey-Kramer HSD post-hoc: $F(6,49)=29.13$; $p < 0.0001$) with D2, D2.B6-*Mop2-D1*, D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD* statistically equivalent to each other ($p=0.92-1.00$) and B6, D2.B6-*Mop2* and D2.B6-*Mop2-P1* statistically equivalent to each other ($p=0.31-0.99$) but with the strains in one group all being statistically different from strains in the other group ($p < 0.0001$ for all comparisons except D2.B6-*Mop2* versus D2.B6-*P1.pD.dD* where $p=0.001$) (Figure 2). There was also a statistically significant strain effect for total fluid intake (ANOVA with Tukey-Kramer HSD post-hoc: $F(6,49)=20.70$; $p < 0.0001$) where B6 was significantly different ($p < 0.0001$) from all other strains, but no other strains were statistically different from each other ($p=0.35-1.00$) (Figure 3). There was a statistically significant strain effect as well for morphine preference score (ANOVA with Tukey-Kramer HSD post-hoc: $F(6,49)=88.02$; $p < 0.0001$) with B6 preference different from D2, D2.B6-*Mop2-D1*, D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD* ($p < 0.0001$ for all comparisons), from D2.B6-*Mop2-P1* ($p=0.013$) and from D2.B6-*Mop2* ($p < 0.01$) (Figure 3). Similar to previous results (Berrettini et al., 1994a), the phenotypic measures of morphine consumption and morphine preference were marked by a strong positive correlation (Pearson $r=0.9316$; $r^2=0.8679$, $p < 0.0001$) whereas quinine consumption and morphine preference were marked by a strong negative correlation (Pearson's $r=-0.9493$; $r^2=0.9011$, $p < 0.0001$).

Overall, strains with the B6 *Mop2* locus (i.e. D2.B6-*Mop2* and D2.B6-*Mop2-P1*) exhibit drinking behavior similar to the B6 strain (Figures 2 and 3). Conversely, strains with the D2 *Mop2* locus (i.e. D2.B6-*P1.pD.dB*, D2.B6-*P1.pD.dD* and D2.B6-*Mop2-D1*) resemble the D2 strain in drinking behavior (Figures 2 and 3). Whereas D2.B6-*Mop2-D1*, D2.B6-*P1.pD.dB*

and D2.B6-*P1.pD.dD* strains show morphine preference scores similar to D2 (0.16 ± 0.08 for D1; 0.21 ± 0.18 and 0.24 ± 0.10 for *P1.pD.dB* and *P1.pD.dD*, respectively, vs. 0.18 ± 0.10 for D2), the D2.B6-*Mop2* and D2.B6-*Mop2-P1* strains exhibit significantly lower morphine preference scores than B6 (0.94 ± 0.01 for B6 vs. 0.72 ± 0.13 for D2.B6-*Mop2* and 0.77 ± 0.11 for D2.B6-*Mop2-P1*) (Figures 1 and 3). This can be explained by the use of quinine in the control bottle, because mice with a D2 genetic background exhibit much weaker aversion to quinine compared to B6 mice (Berrettini et al., 1994b; Lush, 1984). These data suggest that D2.B6-*Mop2-P1* mice retain the B6 gene(s) responsible for the Chr 10 QTL, whereas D2.B6-*Mop2-D1*, D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD* mice do not. This places the *Mop2* QTL gene(s) within the region of Chr 10 between the acromere and the *Lrp11* locus. Thus, the major genetic influence mediating the difference in morphine consumption and/or preference between B6 and D2 mice most likely arises from one or more of 22 genes within the most proximal portion of Chr 10, between the acromere and *Lrp11* (Figure 4).

3.1.2 Gene Expression Analysis

Real-time quantitative RT-PCR gene expression analysis was performed on genes within the introgressed *Mop2* region that have a documented relation to opioid pharmacology (*i.e.* *Oprm1*, *Rgs17*, *Vip*, *Iyd* and *Ppp1r14c*) (Figures 5 and 6). Neither the exon 3-4 nor the custom-designed MOR-1 3'UTR *Oprm1* assays showed statistically significant differences in whole brain expression between naïve B6 and D2 mice (Figure 5A, see “cohort 3”). Similarly, neither the exon 2-3 nor the custom-designed MOR-1 3'UTR *Oprm1* assays showed within-strain differences after morphine treatment in the brain regions examined (striatum, nucleus accumbens [Nacc], midbrain/brainstem [Mb/Bs] or frontal cortex [FCx]; Figure 6A and 6B). However, whereas the *Oprm1* exon 2-3 assay showed no differences between strains at baseline in any brain region (Figure 5A), the custom-designed *Oprm1* MOR-1 3'UTR assay revealed a statistically significant strain difference in FCx with D2 showing higher levels than B6 (Wilcoxon Rank Sum test: $Z=2.259$, $p=0.024$; Figure 5A). *Ppp1r14c* showed no statistically significant differences in either FCx or Mb/Bs between strains at baseline (Figure 5B) or within strains after morphine treatment (Figure 6C). In contrast, *Vip* showed no statistically significant difference between strains at baseline (Figure 5B), but was increased significantly in B6 FCx (Wilcoxon Rank Sum test: $Z=2.647$, $p=0.008$), decreased significantly in B6 Mb/Bs (Wilcoxon Rank Sum test: $Z=-2.802$, $p=0.005$) after morphine treatment, and unchanged in both brain regions of the D2 strain after morphine treatment (Figure 6C). *Iyd* levels were not significantly different in FCx between strains (Figure 5B), but were significantly lower in B6 than D2 Mb/Bs at baseline (Wilcoxon Rank Sum test: $Z=2.123$, $p=0.034$; Figure 5B). *Iyd* levels increased significantly in both brain regions in B6 in response to morphine treatment (Wilcoxon Rank Sum test: $Z=2.482$, $p=0.013$ in FCx; $Z=2.647$, $p=0.008$ in Mb/Bs; Figure 6C), but remained statistically unchanged following morphine treatment of D2 mice (Figure 6C). Finally, *Rgs17* mRNA levels were significantly greater (~1.4X) in D2 than B6 mice at baseline in FCx, Mb/Bs and nucleus accumbens (Nacc), but not in striatum (Wilcoxon Rank Sum test: $Z=2.082$, $p=0.037$ in FCx; $Z=2.327$, $p=0.020$ in Mb/Bs; $Z=2.082$, $p=0.037$ in Nacc; Figure 5B); *Rgs17* mRNA levels in FCx increased in response to morphine treatment in both strains (Wilcoxon Rank Sum test: $Z=2.551$, $p=0.011$ in B6; $Z=-2.327$, $p=0.020$ in D2; Figure 6D). Interestingly, the D2 strain showed a greater fold-increase in *Rgs17* mRNA levels in

response to morphine treatment than the B6 strain (Figure 6D). The effect of morphine pellet implantation on *Rgs17* mRNA levels in B6 mice appears somewhat independent of the μ -opioid receptor as MuKO mice (Sora et al., 1997) bred to the B6 background showed a similar increase in *Rgs17* mRNA levels as the wild-type B6 strain (Figure 6D). In striatum, there was a statistically significant decrease in *Rgs17* mRNA levels in both strains after morphine treatment ($Z=-2.551$, $p=0.011$ in B6; $Z=2.0821$, $p=0.037$ in D2; Figure 6D), an effect that appears dependent on expression of the μ -opioid receptor ($Z=-0.7462$, $p=0.456$ in MuKO; Figure 6D). Finally, in the Nacc, there appears to be an effect of morphine only in the MuKO mice ($Z=-2.452$, $p=0.014$; Figure 6D), a result that is presumably independent of the μ -opioid receptor given the lack of statistically significant changes in *Rgs17* levels in the Nacc of B6 or D2 mice after chronic morphine treatment (Figure 6D).

3.1.3 *Rgs17* Protein Expression Analysis

Because *Rgs17* has the most direct relationship to morphine signaling through the μ -opioid receptor, we determined *Rgs17* protein levels (Figure 7) within the various brain regions examined at the gene expression level (Figures 5 and 6). In contrast to the gene expression results for FCx and Striatum, there were no statistically significant differences in *Rgs17* protein levels in these brain regions between strains after sham-pellet implantation or within-strains comparing sham- to morphine-pellet implanted groups (Figure 7). In agreement with the *Rgs17* gene expression results (Figure 5B), *Rgs17* protein levels were significantly higher in the Mb/Bs and Nacc of D2 sham-pellet implanted animals than in sham-pellet implanted B6 animals (Figure 7). In contrast to no differences in *Rgs17* gene expression (Figure 6D), *Rgs17* protein levels were significantly decreased in the Mb/Bs of D2 mice after morphine-pellet implantation. Finally, there were trends ($p<0.13$) toward statistically significant increased and decreased *Rgs17* protein expression in the Mb/Bs and Nacc, respectively, of B6 mice after morphine-pellet implantation (Figure 7).

4.1 Discussion

4.1.1 *Oprm1*

Results of this study provide new information on the identity and expression of candidate 'quantitative trait genes' (QTG) for the *Mop2* QTL. Consistent with prior studies, *Oprm1* remains a *strong* candidate gene involved in explaining the *Mop2* QTL. In addition, our results suggest that other nearby genes likely play key roles in modulating B6-D2 strain differences in morphine-induced behavioral phenotypes. Support for *Oprm1* as one candidate gene comes from several lines of investigation: 1) it is located under the original QTL peak (Berrettini et al., 1994b; Kozak et al., 1994; Giros et al., 1995), 2) its product, MOR, functions as the main receptor for morphine in the brain (Pasternak, 2001), 3) studies with the MOR-selective antagonist naltrexone suggest that MOR controls the two-bottle choice morphine preference phenotype (Berrettini et al., 1994a), 4) *Oprm1* contains coding and non-coding polymorphisms between B6 and D2 mice that may have functional significance (Doyle et al., 2006). Therefore, results of studies on the D2.B6-*Mop2-P1* (Doyle et al., 2008), and D2.B6-*P1.pD.dB* and D2.B6-*P1.pD.dD* congenic strains (presented here) continue to support the aforementioned hypothesis regarding *Oprm1* as at least one of

the causative gene(s) on Chr 10 that mediates the morphine consumption and preference (*Mop2*) QTL between B6 and D2 mice (Ferraro et al., 2005).

Despite compelling evidence that *Oprm1* (GRCm38/mm10 build genomic base pair position 10:6,788,570-7,038,209) is the morphine preference QTG, there are at least four other genes in the group of 22 remaining within the introgressed region that have some pharmacological relationship to opioids and which therefore also represent candidates for influencing the morphine preference phenotype. Notably, two candidate genes, *Rgs17* and *Ppp1r14c*, influence MOR signal transduction initiated upon morphine binding, whereas two others, *lyd* and *Vip*, might modulate the effects of morphine in other ways.

4.1.2 *lyd*

One of the relevant genes in the interval is *lyd*. Whereas iodothyronine deiodinase (*Dio*) enzyme isoforms are specifically regulated by the selective MOR agonist etonitazene in rats (Baumgartner et al., 1998), the relationship between iodothyronine deiodinase (*lyd*; *a.k.a.* *Dehal1*, 10:3,540,279-3,554,877) and MOR agonists such as morphine is less clear. The *lyd* gene is distinct from the iodothyronine deiodinase (*Dio*) genes that are highly expressed in the thyroid gland and scavenge iodine (Rokita et al., 2010). The role of *lyd* in the thyroid gland is to scavenge iodine from diiodotyrosine (DIT) and monoiodotyrosine (MIT); byproducts of the formation of thyroid hormone from thyroglobulin. Nonetheless, *lyd* is expressed in the frontal cortex, the thalamus and the hippocampus of mouse brain (Allen Brain Atlas; Newrzella et al., 2007) as well as in brain regions of other species (Gaupale et al., 2009) where its role remains unknown. Despite the efficient nature of lysosomal uptake and recycling of MIT and DIT in the thyroid gland (Gnidehou et al., 2004; Rokita et al., 2010), it is controversial whether these halogenated tyrosine byproducts ever gain access to the general circulation from the thyroid gland, and gain access to the brain by crossing the blood-brain barrier (Weinert et al., 1967; Wellby and O'Halloran, 1969). If monoiodinated tyrosine can be transported across the blood-brain barrier, it could act as a potent inhibitor of tyrosine hydroxylase (Goodchild and Brownlee, 1969; Karobath, 1971; Wicker-Thomas and Hamann, 2008). We speculate that *lyd* might relieve tyrosine hydroxylase inhibition by deiodinating monoiodotyrosines that have gained access to the brain and, thus, allow shunting of the resultant tyrosine into the dopamine synthesis pathway. We find that morphine selectively increased *lyd* expression in morphine implanted B6 mice, but not D2 mice (Figure 6C). This might then lead to potentially greater levels of dopamine synthesis in B6 brain regions after morphine, which could enhance the rewarding effects of the drug.

4.1.3 *Vip*

Vasoactive intestinal polypeptide (*Vip*, 10:5,639,218-5,647,614) induces analgesia, perhaps through an interaction with the MOR, yet it reduces acute morphine analgesia (anti-nociception) and blocks the development of tolerance to the analgesic effects of morphine using the radiant heat tail flick assay (Mácsai et al., 1998; Szabó et al., 1998). We find no difference in *Vip* expression between sham pellet-implanted B6 and D2 mice (Figure 5B), but increased and decreased expression of *Vip* in FCx and Mb/Bs, respectively, of morphine pellet-implanted B6 mice compared to D2 mice (Figure 6C). Whether *Vip* plays any role in morphine consumption or preference remains unclear; however, given the decreased

expression of *Vip* in the Mb/Bs in B6 mice after morphine treatment, it is possible that *Vip* might play some role in differential analgesic (hot plate assay) responses between the two strains (Bergeson et al., 2001).

4.1.4 *Ppp1r14c*

The candidates with the strongest biological support after *Oprm1* are regulator of G-protein signaling 17 (*Rgs17*; *a.k.a.* *RgsZ2*, 10:5,825,664-5,922,400) and protein phosphatase 1 (PP1) regulatory (inhibitor) subunit 14c (*Ppp1r14c*; *a.k.a.* kinase-enhanced PP1 inhibitor (*KEPI*), 10:3,366,150-3,464,975). *Ppp1r14c* functions in the signal transduction pathway activated by the MOR and has been reported to be up-regulated mainly in hippocampus and in thalamus following acute and chronic morphine treatment, respectively, but also to a lesser extent in cortex and midbrain after chronic morphine treatment (Liu et al., 2002). In contrast, we observed no statistically significant differences when *Ppp1r14c* mRNA expression was compared between B6 and D2 strains after sham pellet implantation, or within strains after chronic morphine pellet implantation (Figures 5B and 6C). This discrepancy may be a reflection of differences in brain regions studied or methodologies used for measurement of mRNA (Northern blot in (Liu et al., 2002), real-time qRT-PCR used here).

Drgonova et al. (2010) reported strain background effects on morphine-related phenotypes and behaviors in *Ppp1r14c* (*KEPI*) null mutant knockout (KO) mice. Notably, their “original” *KEPI* KO line, on a mixed 129S6/C57BL/6 background, showed differences (between KO and littermate control mice) in the acute tail flick and hot plate tests that were not observed in a “recombinant” *KEPI* KO line (Drgonova et al., 2010) where the *Oprm1* allele was derived from the B6 background strain. The discrepancies in their results were attributed to differences at the *Oprm1* locus (129S6 *versus* B6 allele) rather than to a specific effect of *KEPI* deletion. Nonetheless, whereas the “recombinant” *KEPI* KO line showed no *KEPI* genotype-based difference in development of dependence, as measured by jumps recorded after naloxone-precipitated withdrawal, *KEPI* deletion resulted in faster development of tolerance to morphine measured using the chronic hot plate test, but no difference in spinal analgesia measured using the tail flick test. In addition, their “recombinant” *KEPI* KO line showed a rightward shift in the morphine dose response curve using a conditioned place preference paradigm (Drgonova et al., 2010). Thus, although issues of impure strain backgrounds hamper interpretation of the Drgonova et al. (2010) data, and our data indicate no between-strain or within-strain differences in *Ppp1r14c* (*KEPI*) mRNA expression after chronic sham or morphine pellet implantation, respectively (Figures 5B and 6C), *Ppp1r14c* (*KEPI*) may affect the degree of morphine preference in the two-bottle choice paradigm by virtue of effects on reward and tolerance.

4.1.5 *Rgs17*

Interestingly, the results of *Ppp1r14c* (*KEPI*) deletion on supraspinal nociception (hot plate assay) *versus* spinal analgesia (tail flick assay) were noted to be similar to effects in mice with a *G_z* deletion, the gene encoding the G-protein that is regulated by *Rgs17* (*a.k.a.* *RgsZ2*) (Drgonova et al., 2010; Hendry et al., 2000; Yang et al., 2000; Garzón et al., 2005b). *Rgs17* forms complexes with MOR after morphine treatment, and is thought to play a role in MOR

desensitization (Garzón et al., 2005a, 2005b). *Rgs17* mRNA levels in FCx, Mb/Bs and Nacc at baseline are significantly greater (~1.4X) in D2 compared to B6 mice (Figure 5B); however, in the FCx, a difference in baseline protein expression was not observed (Figure 7). The higher baseline expression of *Rgs17* mRNA (Figure 5B) and Rgs17 protein (Figure 7) in the Nacc and Mb/Bs regions of D2 mice might result in a lower degree of reward from and craving for morphine, respectively, compared to B6 mice. Interestingly, in D2 but not B6 mice, Rgs17 protein levels showed a statistically significant decrease in the Mb/Bs after morphine-pellet implantation (Figure 7). In contrast, in both strains, there was no change in Rgs17 protein expression in the Nacc, Striatum, or FCx after morphine-pellet implantation (Figure 7). The decrease in Rgs17 protein expression in the Mb/Bs of D2 mice after morphine would presumably lead to a slower desensitization of the μ -opioid receptor in the ventral tegmental area, and, thus, subsequently lead to greater craving for morphine. Nonetheless, given the higher expression of Rgs17 protein in the Nacc (which does not change with morphine) (Figure 7), D2 mice might not succumb to cravings, by ingesting more morphine, because the subsequent reward would be diminished in this mouse strain. D2 mice also exhibit a greater relative increase in *Rgs17* mRNA in FCx (Figure 6D); however, this difference was not observed at the protein level (Figure 7). This suggests differential transcriptional regulation between the strains within the FCx, as well as post-transcriptional regulation of Rgs17 expression, but further study is needed. Finally, Zhang et al., (2012) recently reported that polymorphisms within human *RGS17* are associated with multiple drug abuse phenotypes including those involving opioid dependence. Of note, one of the associated polymorphisms, within the promoter region of *RGS17*, led to significantly lower levels of *RGS17* mRNA in Yorubans, but not Caucasians (Zhang et al., 2012). Future work with post-mortem brain tissue will be needed to address whether such polymorphisms that alter *Rgs17* mRNA changes result in differences in Rgs17 protein levels in various brain regions of control and opioid dependent individuals.

4.1.6 Study limitations

Several limitations of these studies should be noted. The mRNA changes delineated herein have not been extended to protein levels. Whereas we conclude that the 13 B6-derived genes remaining in the D2.B6-*P1.pD.dB* congenic strain are not responsible for the *Mop2* QTL, this conclusion is based on a negative result with respect to morphine consumption and preference data. In the absence of another congenic strain (*i.e.* D2.B6-*P1.pB.dD*), we cannot exclude the possibility that some genes or polymorphisms within this region of Chr 10 modulate the *Mop2* QTL through *trans*- or *cis*-regulatory effects, respectively. Because congenic strains were used to assess the behavioral phenotypes, but the parental strains were used in the gene expression analyses, we cannot exclude *trans*-effects of genetic variation within the parental genomes that do not exist within the isogenic congenic strains. Residual heterozygosity between congenic strains was not assessed, and could affect the behavioral phenotypes; however, we note that each of these strains was backcrossed to the parental inbred strain for greater than 10 generations making any residual heterozygosity, in theory, quite limited. Whereas we have not assessed directly the development of morphine dependence for each strain used in the drinking experiments, B6 mice exhibit clear signs of dependence in this test, even death by presumed overdose in 5% of B6 mice (Berrettini et al, 1994b). We are unsure of the level of dependence achieved in the D2 mice and other strains

that do not consume large quantities of morphine in the drinking experiment. Nonetheless, after 5-day morphine pellet implantation, both the B6 and D2 strains exhibit clear signs of dependence as measured by withdrawal (jumping), after naloxone injection (data not shown).

The parental strains (B6 and D2) have been extensively studied with respect to various other morphine phenotypes including anti-nociception and analgesia, however, the various congenic and subcongenic strains described herein have not been assessed to the same extent (*i.e.* the D2.B6-*Mop2-P1*, D2.B6-*Mop2-D1* or D2.B6-*P1-pD.dB* congenic strains). However, several of the strains were shared with other laboratories and some studies on these congenic strains have been reported in the literature. Particularly with respect to anti-nociception, Tapocik et al (2009) found that “B6 and B6D2 genotypes were significantly more sensitive than the D2 and D2B6 mice. The B6 and B6D2 genotypes did not differ from each other nor did the D2 and D2B6 genotypes. Therefore, baseline pain sensitivity was not affected by the introgression of the proximal region of Chr 10.” The B6D2 and D2B6 strains (strain D2.B6-*Mop2* in the current manuscript) used in Tapocik et al (2009) were derived from breeders of our initial reciprocal congenic strains (B6.D2-*Mop2* and D2.B6-*Mop2*; studied for morphine preference in Ferraro et al, 2005) that were sent from our mouse colony. Likewise, Bergeson et al (2001) found multiple QTL for anti-nociception, including one on proximal Chr 10, but state that “In the S3, the HMOR mice received half the dose of the LMOR (6 vs 12 mg/kg, respectively), yet the anti-nociception scores were closely similar (17.0 ± 8.3 vs 21.7 ± 6.7 , respectively, *n.s.*). This shows that the S3 HMOR mice were about twice as sensitive to morphine as LMOR in terms of the equieffective dose.” Given that Bergeson et al (2001) found additional QTL affecting anti-nociception, that they bred for only 3 generations rather than making N10 generation reciprocal congenics (Ferraro et al, 2005), and that Tapocik et al (2009) found no difference between the parental and Chr10 congenic strains in anti-nociception, these data suggest modulation of the proximal Chr10 anti-nociception phenotype by other regions of the parental genome(s). Tapocik et al (2009) also studied analgesia with the reciprocal congenic strains (Ferraro et al, 2005), and found that “as was true for baseline nociception, introgression of the proximal Chr10 region did not alter acute sensitivity to the analgesic effects of morphine; the B6 and B6D2 genotypes did not differ from each other nor did the D2 and D2B6 genotypes. This was a surprising finding considering bQTL analyses have mapped the proximal chromosome region of 10 to morphine analgesia (Belknap et al., 1995) and other morphine-related behaviors (Berrettini et al., 1994b; Ferraro et al., 2005).” However, the reciprocal congenic strains (Ferraro et al, 2005) did differ in their development of tolerance to the analgesic effects of morphine (Tapocik et al, 2009). This again suggests modulation of the proximal Chr10 QTL phenotypes by other regions of the parental genome(s). In addition, it suggests differences in acute *versus* chronic effects of morphine in the strains.

4.1.7 Conclusions

In summary, we have narrowed a QTL, *Mop2*, controlling morphine consumption and preference in a two-bottle choice drinking paradigm, to a genomic interval of ~7.6 Mbp on Chr 10. The interval contains 22 genes including several notable biological candidates, including *Oprm1*. Given the potential for other genes in the newly refined *Mop2* interval to

affect the pharmacology of opioids, as well as their differential regulation by morphine, it is possible that multiple genetic variants contribute to the difference in morphine preference between B6 and D2 mice. Further refinement of the QTL interval will be required to prove definitively the identity of the QTG(s) within the *Mop2* QTL.

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Abbreviations

QTL	quantitative trait loci
QTG	quantitative trait gene
Mop2	morphine preference 2 QTL
B6	C57BL/6J mouse
D2	DBA/2J mouse
MOR	μ-opioid receptor
MuKO	MOR knockout mouse
Chr	chromosome
cM	centiMorgan
SNP	single nucleotide polymorphism
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
Mbp	Million base pairs
SEM	standard error of the mean
HSD	Honestly Significant Difference

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Highlights

- Chr 10 morphine preference *Mop2* QTL refined to 22 candidate genes.
- *Ppp1r14c* mRNA does not differ among morphine treated C57BL/6 and DBA/2 mice
- Differential mRNA expression of *Iyd* and *Vip* among C57BL/6 and DBA/2 mice
- Morphine increases *Rgs17* mRNA, but not Rgs17 protein in frontal cortex of DBA/2 and C57BL/6 mice
- Rgs17 protein levels differ between C57BL/6 and DBA/2 mice in Midbrain/Brainstem and Nucleus accumbens regions

Figure 1. Schematic of Chr 10 in congenic strains tested in the two-bottle choice paradigm
The mouse strains presented in this report is shown. Genes and DNA microsatellite markers that were genotyped for these studies are given above the Chr. Their relative positions (in Mbp from the acromere) on the physical map (genome.ucsc.edu; GRCm38/mm10) are given below the Chr (see also Figure 4). Black reflects regions contributed by the B6 parental strain whereas grey regions reflect contributions from the D2 parental strain. White regions indicate regions of undetermined origin. Genes targeted for expression analysis are highlighted in bold and indicated with an asterisk. Note that strain D2.B6-P1.pD.dD (littermate controls) mice are essentially genetically identical to parental D2 mice. The two-bottle choice morphine preference scores for the various strains tested are indicated to the right of the figure.

Figure 2. Morphine and quinine consumption by Chr 10 congenic mice

Mouse strains (see Fig 1 for key) were tested in the two-bottle choice paradigm with saccharin plus morphine or quinine as described in section 2.1.3. The mean (+SEM) consumption over days 9-12 is shown. ANOVA with Tukey's HSD *post-hoc*: * $P < 0.0001$ vs. all other strains; # $p < 0.01$ vs. strains D2, D2.B6-D1, P1-pD.dB and P1-pD.dD; @ $p < 0.05$ vs. strains D2, D2.B6-D1, P1-pD.dB and P1-pD.dD for morphine consumption. & $P < 0.0001$ vs. strains D2, D2.B6-D1, P1-pD.dB and P1-pD.dD; \$ $p = 0.001$ vs. strain P1-pD.dD for quinine consumption. Note that strain P1-pD.dD (littermate controls) mice are essentially genetically identical to parental D2 mice. Data for strains B6, D2, D2.B6-P1, D2.B6-D1 and D2.B6-Mop2 were originally published in and are reproduced from (Doyle et al., 2008).

Figure 3. Morphine preference and total fluid intake by Chr 10 congenic mice

Mouse strains (see Fig 1 for key) were tested in the two-bottle choice paradigm with saccharin plus morphine or quinine as described in section 2.1.3. The mean (+SEM) preference scores and total fluid intake over days 9-12 is shown. ANOVA with Tukey's HSD *post-hoc*: * $P < 0.0001$ vs. all other strains for total fluid intake. # $P < 0.0001$ vs. strains D2, D2.B6-D1, P1-pD.dB and P1-pD.dD for morphine preference. @ $P < 0.02$ vs. strains D2.B6-P1 and D2.B6-Mop2 for morphine preference. Note that strain P1-pD.dD (littermate controls) mice are essentially genetically identical to parental D2 mice. Data for strains B6, D2, D2.B6-P1, D2.B6-D1 and D2.B6-Mop2 were originally published in and are reproduced from (Doyle et al., 2008).

Figure 4. Chr 10 genes within the introgressed congenic critical interval

UCSC genome browser (genome.ucsc.edu; GRCm38/mm10) screen shot showing the 22 candidate genes remaining within the introgressed region of D2.B6-*Mop2-P1.pD.dB* congenic mice. The scale and base pair position of each gene is shown along the top of the image. SNPs existing among *all* (*i.e.* not just B6 and D2) sequenced mouse strains are shown at the bottom of the image. Color coding of SNPs is by conventional (default) settings used by the UCSC genome browser.

Figure 5. Baseline gene expression differences between B6 and D2 mice

B6 gene expression levels were used as an arbitrary comparator, and were set to a value of 1.0. The relative level of target gene expression ($2^{-(C_t)}$ method) is given with error bars indicating the lower ($2^{-(C_t + S.D.)}$) and upper ($2^{-(C_t - S.D.)}$) bounds calculated from the standard deviations (S.D.). The p -values were determined using the Wilcoxon Rank Sum test on the means and standard deviations of the C_T values. (A) *Oprm1* gene expression was measured in either whole brain [Wb] of naïve mice (cohort 3) or in the brain regions indicated (striatum, nucleus accumbens [Nacc], midbrain/brainstem [Mb/Bs], frontal cortex [FCx]) of sham pellet-implanted mice (cohorts 1 and 2) using either the exon 2-3, exon 3-4 or custom MOR-1 3'UTR Taqman assay. * $p < 0.05$. (B) *Rgs17*, *Vip*, *Ild* and *Ppp1r14c* gene expression was measured in the brain regions indicated of sham pellet-implanted mice (cohorts 1 and 2) using the corresponding Taqman assays. * $p < 0.05$. Note that *Rgs17* data are from cohort 1 and *Vip*, *Ild* and *Ppp1r14c* data are from cohort 2. (See Table 1 for the numbers of mice in each strain cohort.)

Figure 6. Gene expression differences between sham or morphine pellet-implanted B6 or D2 mice

Sham pellet-implanted gene expression levels were used as an arbitrary comparator, and were set to a value of 1.0. The relative level of target gene expression ($2^{-(C_T)}$ method) is given with error bars indicating the lower ($2^{-(C_T+S.D.)}$) and upper ($2^{-(C_T-S.D.)}$) bounds calculated from the standard deviations (S.D.). The p -values were determined using the Wilcoxon Rank Sum test on the means and standard deviations of the C_T values. *Oprm1* gene expression was measured in the brain regions indicated (striatum, nucleus accumbens [Nacc], midbrain/brainstem [Mb/Bs], frontal cortex [FCx]) of sham or morphine pellet-implanted (A) B6 mice (cohorts 1 and 2) or (B) D2 mice (cohorts 1 and 2) using either the exon 2-3 or custom MOR-1 3'UTR Taqman assay. (See Table 1 for the numbers of mice in each strain cohort). (C) *Vip*, *Idy* or *Ppp1r14c* gene expression was measured in the brain regions indicated (striatum, nucleus accumbens [Nacc], midbrain/brainstem [Mb/Bs], frontal cortex [FCx]) of sham or morphine pellet-implanted B6 mice (cohort 2) or D2 mice (cohort 2) using the corresponding Taqman assays. * $p < 0.05$, ** $p < 0.01$. (D) *Rgs17* gene expression was measured in the brain regions indicated (striatum, nucleus accumbens [Nacc], midbrain/brainstem [Mb/Bs], frontal cortex [FCx]) of sham or morphine pellet-implanted B6 mice (cohort 2) or D2 mice (cohort 2) using the corresponding Taqman assay. * $p < 0.05$. (See Table 1 for the numbers of mice in each strain cohort).

Figure 7. Rgs17 protein expression in various brain regions of sham- and morphine-pellet implanted B6 or D2 mice

Total protein from the indicated brain regions was analyzed for Rgs17 and β -actin protein expression by infra-red fluorescent immunoblot. Band intensities were measured and the β -actin signal was used to normalize the Rgs17 signal in each lane. The averages and standard deviations for each group (N=3) are shown in arbitrary units. Statistical significance was determined by one-way ANOVA followed by the non-parametric Wilcoxon rank sum test. An asterisk (*) indicates $p < 0.05$ for the comparisons indicated by the lines.

Table 1

Gene Expression Analysis: Numbers of mice in each strain cohort

Gene	Strain/Brain Region/Condition
<i>Oprm1</i> exon 2-3 2nd cohort	B6 FCx Sham (n=6) B6 FCx Morphine (n=6) D2 FCx Sham (n=8) D2 FCx Morphine (n=7) B6 Mbn/Brstem Sham (n=6) B6 Mbn/Brstem Morphine (n=6) D2 Mbn/Brstem Sham (n=8) D2 Mbn/Brstem Morphine (n=7)
<i>Oprm1^a</i> exon 2-3 1st cohort	B6 Mbn/Brstem Sham (n=4) B6 Mbn/Brstem Morphine (n=7) D2 Mbn/Brstem Sham (n=5) D2 Mbn/Brstem Morphine (n=4) B6 Striatum Sham (n=4) B6 Striatum Morphine (n=7) D2 Striatum Sham (n=5) D2 Striatum Morphine (n=3) B6 Nacc Sham (n=4) B6 Nacc Morphine (n=7) D2 Nacc Sham (n=5) D2 Nacc Morphine (n=4)
<i>Oprm1</i> exon 3-4 3rd cohort	B6 Whole Brain naïve (n=3) D2 Whole Brain naïve (n=3)
<i>Oprm1</i> MOR-1 3'UTR 2nd cohort	B6 FCx Sham (n=6) B6 FCx Morphine (n=6) D2 FCx Sham (n=8) D2 FCx Morphine (n=7) B6 Mbn/Brstem Sham (n=6) B6 Mbn/Brstem Morphine (n=6) D2 Mbn/Brstem Sham (n=8) D2 Mbn/Brstem Morphine (n=7)
<i>Oprm1</i> MOR-1 3'UTR 3rd cohort	B6 Whole Brain naïve (n=3) D2 Whole Brain naïve (n=3)
<i>Rgs17^a</i> 1st cohort	B6 FCx Sham (n=4) B6 FCx Morphine (n=7) D2 FCx Sham (n=5) D2 FCx Morphine (n=4) B6-MUKO FCx Sham (n=4) B6-MUKO FCx Morphine (n=6) B6 Mbn/Brstem Sham (n=4) B6 Mbn/Brstem Morphine (n=7) D2 Mbn/Brstem Sham (n=5) D2 Mbn/Brstem Morphine (n=4) B6-MUKO Mbn/Brstem Sham (n=4) B6-MUKO Mbn/Brstem Morphine (n=6) B6 Striatum Sham (n=4) B6 Striatum Morphine (n=7) D2 Striatum Sham (n=5) D2 Striatum Morphine (n=4) B6-MUKO Striatum Sham (n=4) B6-MUKO Striatum Morphine (n=6) B6 Nacc Sham (n=4) B6 Nacc Morphine (n=7) D2 Nacc Sham (n=5) D2 Nacc Morphine (n=4) B6-MUKO Nacc Sham (n=4) B6-MUKO Nacc Morphine (n=6)
<i>Vip</i> 2nd cohort	B6 FCx Sham (n=5) ^b B6 FCx Morphine (n=6) D2 FCx Sham (n=8) D2 FCx Morphine (n=7) B6 Mbn/Brstem Sham (n=6)

Gene	Strain/Brain Region/Condition
	B6 Mbn/Brstem Morphine (n=6) D2 Mbn/Brstem Sham (n=8) D2 Mbn/Brstem Morphine (n=7)
<i>lyd</i> 2nd cohort	B6 FCx Sham (n=6) B6 FCx Morphine (n=6) D2 FCx Sham (n=8) D2 FCx Morphine (n=7) B6 Mbn/Brstem Sham (n=5) ^b B6 Mbn/Brstem Morphine (n=6) D2 Mbn/Brstem Sham (n=8) D2 Mbn/Brstem Morphine (n=7)
<i>Ppp1r14c</i> 2nd cohort	B6 FCx Sham (n=6) B6 FCx Morphine (n=6) D2 FCx Sham (n=8) D2 FCx Morphine (n=7) B6 Mbn/Brstem Sham (n=6) B6 Mbn/Brstem Morphine (n=6) D2 Mbn/Brstem Sham (n=8) D2 Mbn/Brstem Morphine (n=7)

^a Different cohorts of mice were used for *Rgs17* and *Oprm1* (exon 2-3) assays.