2017

Fine mapping of a major quantitative trait locus mediating multiple opioid addiction behaviors

https://hdl.handle.net/2144/23822

Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

FINE MAPPING OF A MAJOR QUANTITATIVE TRAIT LOCUS MEDIATING MULTIPLE OPIOID ADDICTION BEHAVIORS

by

ALEXANDER LUONG
B.S., University of California, San Diego, 2014

Submitted in partial fulfillment of the requirements for the degree of Master of Science
2017
ACKNOWLEDGMENTS

I would like to thank Dr. Bryant for allowing me this opportunity to participate in his laboratory. I would like to also thank Dr. Goldberg for her mentorship throughout this process.
FINE MAPPING OF A MAJOR QUANTITATIVE TRAIT LOCUS MEDIATING MULTIPLE OPIOID ADDICTION BEHAVIORS

ALEXANDER LUONG

ABSTRACT

Opioid addiction is a growing epidemic with no known genetic basis. Mice represent a valuable tool that can be used to better understand the genetic components of opioid addiction by studying opioid induced behaviors such as locomotor activity. The two closely related C57BL/6 substrains, C57BL/6J (B6J) and C57BL/6NJ (B6NJ), exhibit limited genetic diversity, yet display phenotypic differences when under the influence of oxycodone. Quantitative trait locus (QTL) mapping, a discovery based approach to identifying genomic regions underlying statistical variation in complex traits, was used to identify a locus on distal chromosome 1 for oxycodone-induced locomotor activity and withdrawal. F\textsubscript{2} offspring from a cross utilizing these substrains that are homozygous B6J across the chromosome 1 QTL compared to offspring that are heterozygous display the same phenotypic differences as the parental strains, namely oxycodone-induced locomotor activity. F\textsubscript{2} mice were selected based on distal chromosome 1 genotypes and backcrossed to parental strain C57BL/6J to fine map the QTL interval. Through family analysis, regions proximal to 167 Mb and distal to 187 Mb have been ruled out as containing the QTL mediating oxycodone-induced locomotor activity. Future studies should employ this same technique to fine map the QTL.
mediating oxycodone withdrawal in order to differentiate whether it is one locus or two loci controlling these oxycodone induced behaviors.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>READER APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>17</td>
</tr>
<tr>
<td>METHODS</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>26</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>34</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>39</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>44</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SNP Assays Across Distal Chromosome 1</td>
<td>25</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Place Conditioning Chamber and CPP Protocol</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Creating an $F_2$ population</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>$B6J/B6NJ$ Parental Strain Distance Traveled</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Locus on Chromosome 1 Affects OXY Sensitivity</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Locus on Chromosome 1 Affects OXY Withdrawal</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>QTL for OXY-Induced Locomotor Activity and OXY Withdrawal</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Abbreviated Two Day Paradigm</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Fine Mapping a QTL Mediating OXY-induced Locomotor Activity using Congenic Strains</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>OXY-Induced Locomotor Activity (Spins)</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>OXY-Induced Locomotor Activity (Rotations)</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>Rule out Region Distal to 187 Mb</td>
<td>31</td>
</tr>
<tr>
<td>12</td>
<td>Rule out Region Proximal to 167 Mb</td>
<td>33</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>B6J</td>
<td>C57BL/6J</td>
<td></td>
</tr>
<tr>
<td>B6NJ</td>
<td>C57BL/6NJ</td>
<td></td>
</tr>
<tr>
<td>cM</td>
<td>Centimorgan</td>
<td></td>
</tr>
<tr>
<td>CPP</td>
<td>Opioid maintenance therapy</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>Day 2</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>Day 4</td>
<td></td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
<td></td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association studies</td>
<td></td>
</tr>
<tr>
<td>i.p.</td>
<td>Intra-peritoneal</td>
<td></td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of odds</td>
<td></td>
</tr>
<tr>
<td>OMT</td>
<td>Opioid maintenance therapy</td>
<td></td>
</tr>
<tr>
<td>OXY</td>
<td>Oxycodone</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
<td></td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
<td></td>
</tr>
<tr>
<td>RCC</td>
<td>Reduced complexity cross</td>
<td></td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
<td></td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
<td></td>
</tr>
<tr>
<td>SSLP</td>
<td>Simple sequence length polymorphism</td>
<td></td>
</tr>
<tr>
<td>SAL</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

Background

Worldwide, opioid dependence is the largest contributor to years lost in one’s life due to a disease (Degenhardt et al. 2013). In 2014, 49.2% of all drug abuse deaths in the United States were attributable to opioid abuse (Laslo et al. 2017). From 2007 to 2013, the number of users in the United States increased from 373,000 to 681,000 (Laslo et al. 2017). Recently, the state of Massachusetts has declared a state of public emergency in regards to the rising opioid epidemic (Department of Public Health 2014). “Opioid” is a general term used to classify a group of highly addictive drugs, including both illicit (i.e. heroin) and prescription drugs, such as oxycodone, fentanyl, and morphine. Opioid use disorder is classified by the Diagnostic and Statistical Manual of Mental Disorders as a chronic condition that is defined in part by the continued use opioids despite negative consequences in areas of life including one’s health, work, school, or home (American Psychiatric Association, 2013)

The current forms of treatment for opioid addiction treat only symptoms or consequences of the disease rather than the disease itself. One form of symptom treatment is opioid maintenance treatment (OMT), which is the daily use of an opioid agonist, (i.e. methadone), to limit cravings and withdrawal symptoms. Methadone has been widely accepted as a standard form of treatment for opioid dependence by healthcare providers since the 1960s (Laslo et al. 2017). As a consequence, users become dependent on a different synthetic opioid, while still facing risks of harmful respitatory depression and potential addiction/overdose (Laslo et al. 2017). Another form of symptom treatment to
alleviate physiological effects of withdrawal involves the use of clonidine, an alpha 2
adrenergic receptor agonist (Gowing et al. 2016). While it does not help to satiate opioid
cravings, it helps reduce physical symptoms of withdrawal such as anxiety, agitation, and
muscle spasms (Capino et al. 2016).

A major life-threatening consequence of opioid use is overdose, which is
currently treated with the use of opioid antagonists, such as naloxone or naltrexone.
These drugs are non-selective, specific opioid receptor antagonist (Lobmaier et al. 2016).
Again, this form of treatment does not treat the disease itself but rather a side effect due to
the disease. It is clear that new forms of treatment, ones that tackle the disease rather
than the symptoms of addiction, must be developed.

**Genetics of Opioid Addiction**

Opioid addiction is a complex disease that involves a multitude of factors
including one’s susceptibility to the disease as well as environmental factors. The
interaction of three major factors that contribute to the pathogenesis of opioid addiction:
genetic factors (Kendler et al. 2003; Tsuang et al. 1996; Tsuang et al. 1998),
drug-induced neurobiological changes, and influence of other environmental factors besides
drug exposure (e.g. early life trauma) (Nielsen and Kreek 2012). Based on twin, family,
and adoption studies, it has been shown that opioid abuse/dependence is 43-60%
heritable but this genetic component is not yet well understood (Ho et al. 2010). While
genome wide association studies (GWAS) have been conducted in humans, there has
been little success in identifying genes underlying drug addiction due to large number of
statistical tests required and the small effect sizes that can be expected from the
correlation of common variants to complex diseases. A human GWAS examining
500,000 SNPs with a p = 0.05 could potentially give 25,000 false positives (Ho et al.
2010). As such, genome-wide statistical significance of p < 1 x 10^{-7} is typically used (Ho
et al. 2010), however, that requires extremely large population sizes to achieve
significance. For example, a recent human GWAS which identified 108 significant loci
associated with schizophrenia involved 36,989 cases and 113,075 controls
(Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014).

Mouse Models to Study Genetics of Addiction

While drug addiction is purely a human disease, mammalian models are a
valuable tool that can be used to study addiction relevant phenotypes. Mice in particular
are especially valuable because 99% of mice genes have a direct human counterpart (Kile
and Hilton 2005). In addition, there are many practical reasons that make the use of mice
advantageous- mice have shorter generation times than other mammals and can be bred
easily, allowing for studies that require large population numbers. Addiction is a
complex disease that involves multitudes of variables, including environmental variables
that are difficult to control for in humans. In studies utilizing mammalian models, there
is greater control over environmental factors such as housing, food, temperature,
humidity, and light/dark cycles.

All drugs of abuse share a common circuitry in the brain’s limbic system, with
each drug activating specific dopaminergic transmissions to produce their effects (Nestler
2005). In particular, opioids cause dopamine release in the nucleus accumbens (Di Chiara and Imperato 1988), an important area for reward learning in the brain (Flagel et al. 2011). Opioids bind to mu opioid receptors on GABA-ergic interneurons in the ventral tegmental area (VTA), causing hyperpolarization (Johnson and North 1992). This leads to a decrease in synaptic input that causes excitation of dopamine cells via disinhibition, leading to increased dopamine release. The nucleus accumbens is involved in both locomotor activity and drug reward learning (Di Chiara and Imperato 1988; Wise and Bozarth 1987). Therefore, locomotor activity (i.e. drug sensitivity) can sometimes be used as a proxy to identify common genetic factors involved in both locomotor activity and drug reward. Sensitivity to the locomotor stimulant response to opioids is heritable (Phillips et al. 2008; Belknap et al. 1998; Oliverio et al. 1975; Gill and Boyle 2008; Philip et al. 2010). Furthermore, variation in acute locomotor sensitivity in rodents and subjective drug euphoria in humans can predict susceptibility to drug-self administration as well as risk for drug dependence (Piazza et al. 2013; Haertzen et al. 1983). Therefore, drug sensitivity and drug reward share at least some common genetic factors. Furthermore, drug liking is predicative of future abuse (Haertzen et al. 1983). Thus, understanding the genetic basic of opioid addiction would prove crucial in developing new treatments aimed at curbing opioid addiction.

**Conditioned Place Preference**

Mammalian behavioral models can be used to study the rewarding (drug liking) effects of drugs (Koob and Volkow 2010). In the conditioned place preference (CPP)
model, a mouse is confined to a chamber with two different “environments”. On designated training days, the mouse is restricted to one environment and either given drug or saline. After training, the mouse is allowed access to both environments and tested for association of drug with the drug-paired environment. Preference for the drug-paired side is thought to represent the rewarding properties of the drug (conditioned place preference, CPP) (Bryant et al. 2014; Tzschentke 2007). With this model, drug-induced locomotor activity and locomotor sensitization can be measured on training days. CPP assessment can be measured both with and without the influence of drug, to determine state-dependent and state-independent preferences (Tzschentke 2007).
(A) Two-chamber design with each chamber differentiated by floor texture. Video recording from above and AnyMaze software used for video tracking allows for detection of numerous drug-related behaviors, such as rotations (Rot., defined as completion of unidirectional, circular sequence across four zones 1-2-3-4 or 3-2-1-4).

(B) Schematic of nine day place conditioning protocol. Mice are allowed access to both sides of chamber and assessed for initial preference on Day 1. Days 2-5 are training days, where mice receive either drug or saline injections. Days 6-7 are consolidation days, with mice being left undisturbed in home cages. On Day 8 mice are assessed for final preference in drug free state. On Day 9 mice are assessed for state-dependent preference. Rot. = rotation, D = day, Pref. = preference, SAL = saline, CPP/CPA = conditioned place preference, conditioned place aversion (Kirkpatrick and Bryant 2014)
Quantitative Trait Locus Mapping

Quantitative trait locus (QTL) mapping is an unbiased, discovery-based approach to identifying genomic regions and ultimately genetic variants underlying statistical variation in complex traits. This process identifies a QTL, an area of the genome that is associated with the phenotypic expression of complex traits (Tarantino and Eisener-Dorman 2012). Typically, QTL mapping is used to identify genotypic variation responsible for phenotypic variation in inbred strains (Tarantino and Eisener-Dorman 2012). An inbred strain is a mouse strain that has undergone brother-sister mating for at least 20 generations, resulting in genetically identical offspring. Each individual within an inbred strain is genetically identical and thus, any phenotypic variation within an inbred strain is caused by environmental factors and any variation between inbred strains is caused by genetic factors. The C57BL/6 inbred mouse strain is the most widely used strain in biomedical research (Bryant 2011). Two of its substrains, C57BL/6J (B6J) and C56BL/6NJ (B6NJ) are nearly genetically identical, yet display phenotypic variation for numerous complex traits. When two inbred strains are crossed, the offspring will be heterozygous at every allele (F₁ generation) (Figure 2). F₁ offspring are then bred together to create an F₂ generation. The genetic variation between strains can then be tracked using a set of polymorphic markers that distinguish the two strains. Over time, the type of markers used has changed from restriction fragment length polymorphisms (RFLPs) to simple sequence length polymorphisms (SSLPs) to the more high throughput single nucleotide polymorphisms (SNPs) (Tarantino and Eisener-Dorman 2012). Once genotypes and phenotypes have been collected, a statistical analysis can be run and a
logarithm of odds (LOD) score is generated. The score is a measurement of the strength of statistical association between phenotypic variance and genetic variance that is in linkage disequilibrium with the causal variant(s). Essentially, the score compares the likelihood of obtaining test results due to linkage vs. obtaining the same results due to random chance. A score greater than 3 generally translates to $p = 0.05$ (Nyholt 2000), and is considered evidence for significant linkage. It indicates 1000 to 1 odds that the linkage observed did not occur by chance.

**Fine Mapping a Quantitative Trait Locus**

Standard QTL crosses, such as an F$_2$ intercross, usually yield genomic regions over 20 Mb due to the relatively low number of recombination events in these populations (Tarantino and Eisener-Dorman 2012). A region this large could contain hundreds of genes and thousands of polymorphisms, making it difficult to identify the genetic variant responsible for the QTL. Identification of the quantitative trait gene(s) (QTGs) typically requires refinement of the QTL interval. Narrowing of this interval involves the use of specialized populations, namely congenic strains. Congenic strains are made from repeatedly backcrossing an allele from a donor strain onto a recipient strain until only a small region of the QTL-containing donor strain chromosome is transferred into the recipient strain background (Tarantino and Eisener-Dorman 2012). This process usually takes at least 13 generations.

Drawbacks of using congenic strains include the possibility of QTL effects disappearing during the construction of the congenic strain. This would result if the QTL
is removed from a heterogeneous background, eliminating epistatic interactions that increase QTL effect size (Tarantino and Eisener-Dorman 2012). A loss of QTL signal could also result if the QTL was actually composed of a group of smaller effect size QTLs that appear as a single peak in a F2 population (Tarantino and Eisener-Dorman 2012; Bryant et al. 2012). The major drawback of fine mapping via congenic strains is the time- and labor-intensive nature of backcrossing necessary to create a truly congenic strain, where the genome is isogenic at every point except for at the QTL interval.
Inbred strains (such as C57BL/6J) are created from brother-sister mating for at least 20 generations, resulting in genetically identical offspring homozygous at every allele. Two inbred strains are then bred to create an F_1 population heterozygous at every allele. Non-sibling F_1 mice are bred to create an F_2 population which contain one or two copies from each parental strain.
Identification of A QTL on Distal Chromosome 1 for Oxycodone Sensitivity and Withdrawal Using the Reduced Complexity Cross

Our laboratory has recently identified phenotypic behavioral differences in the closely related C57BL/6J (B6J) and C57BL/6NJ (B6NJ) mice for opioid antagonist aversion behaviors (Kirkpatrick and Bryant 2014) and opioid agonist behaviors (Goldberg et al. 2017). The B6J and B6NJ substrains exhibit significant differences in oxycodone (OXY) sensitivity, specifically distance traveled on Day 2 (D2) and 4 (D4) of the OXY place conditioning paradigm (Figure 3). To determine the genetic component responsible for the observed differences, our laboratory generated an F2 cross of B6J and B6NJ, denoted as the reduced complexity cross (RCC). We identified a region of distal chromosome 1 that was responsible for variation in OXY behavioral sensitivity (Figure 4). Similar to the parental strains, we identified that F2 mice that were homozygous B6J at this locus exhibited increased OXY-induced locomotor activity (i.e. sensitivity) compared to those that were heterozygous or homozygous B6NJ (Figure 4). Specifically, F2 mice that were homozygous B6J displayed increased spins, rotations, and distance traveled on Days 2 and 4 (first and second exposure to OXY in the place conditioning paradigm) (Figure 4). Interestingly, a QTL for opioid withdrawal utilizing the elevated plus maze (EPM), co-mapped to this same region (Figure 5/6). Opioid withdrawal in mice leads to increased open arm time in the elevated plus maze (Hofford et al. 2009; Hodgson et al. 2008; Buckman et al. 2009). By utilizing the RCC, we greatly decreased the potential number of variants responsible for variation in the behavior, however we were still left with an interval of 163-190 Mb. Also interestingly, D2 OXY-induced
locomotor activity appears to exhibit Mendelian inheritance, meaning that one major locus likely underlies parental strain variation. Strain accounts for 25.25% variance in phenotype in parental strains and parental strain genotype at this QTL locus accounts for 19.16% variance in phenotype in the F\(^2\) population. Furthermore, a similar effect size was exhibited between the parental strains and F\(^2\) mice that are homozygous for the J vs. N alleles, providing further support for a Mendelian inheritance. A Mendelian inheritance eliminates concerns regarding the potential for epistatic loci on other chromosomes and suggests that we can immediately begin fine mapping this locus by backcrossing F\(^2\) mice that are recombinant within the QTL interval.
Parental strain B6J displayed increased locomotor activity compared to B6NJ under the influence of OXY. Strains displayed no differences under SAL control.

SAL = saline, OXY = oxycodone, B6J = C57BL/6J, B6NJ = C57BL/6NJ
(A) QTL plots for distance traveled, spins, and rotations on Days 2 and 4.
(B) Effect plot showing effect of genotype at QTL peak on Day 2 distance traveled in OXY vs. SAL treated F$_2$ mice.
(C) - (G) Effect plots showing effect of genotype at QTL peaks for associated phenotypes on Days 2 or 4.

BB = homozygous B6J, BN = heterozygous B6J/B6NJ, NN = homozygous B6NJ, LOD = logarithm of odds, cM = centimorgan, QTL = quantitative trait locus, SAL = saline, OXY = oxycodone (Goldberg et al. 2017)
Figure 5: Locus on Chromosome 1 Affects OXY Withdrawal

(A) QTL plot for open arm time in elevated plus maze.
(B) Effect plot showing effect of genotype on time spent in open arm of elevated plus maze in OXY vs. SAL treated F$_2$ mice.

BB = homozygous B6J, BN = heterozygous B6J/B6NJ, NN = homozygous B6NJ, LOD = logarithm of odds, cM = centimorgan, QTL = quantitative trait locus, SAL = saline, OXY = oxycodone (Goldberg et al. 2017)
Figure 6: QTL for OXY-Induced Locomotor Activity and OXY Withdrawal

QTL plots for QTL(s) mediating OXY-induced locomotor activity and OXY withdrawal peaked in same region on distal chromosome 1. LOD scores over 3 are considered evidence of significant linkage (Nyholt 2000).

$LOD = \log$arithm of odds, $QTL = \text{quantitative trait locus}$, $OXY = \text{oxycodone}$, $D2 = \text{day 2}$ (Goldberg et al. 2017)
OBJECTIVES

Fine Map Distal Chromosome 1 using F2 Recombinants

The closely related C57BL/6J and C57BL/6NJ strains of mice are two inbred substrains from the C57BL/6 family. Recently, our laboratory has identified phenotypic variation in OXY-induced locomotor activity in the OXY conditioned place preference assay (OXY-CPP, Figure 3). Specifically, the strains showed variation in Day 2 and Day 4 distance traveled, rotations, and spins. Our laboratory mapped a QTL to distal chromosome 1 that is responsible for the variation in behavior. While QTLs identified using an RCC are usually quite large (tens of MBs), the advantage is that the number of polymorphic genes and functional variants responsible for trait variation are several orders of magnitude lower (Kirkpatrick et al. 2016; Kumar et al. 2013). For example, the DBA/2J mouse line and C57BL/6J differ by about 4 million SNPs (Keane et al. 2011) while C57BL/6J and C57Bl/6NJ differ by about 10,000 SNPs (Simon et al. 2013). Making the same comparison within the QTL interval on distal chromosome 1 (172 – 178 Mb), 23,723 SNPs within genes differ between DBA/2J and B6J vs. 83 SNPs between B6J and B6NJ (Keane et al. 2011; Yalcin et al. 2011).

To further fine map and narrow the QTL interval, we selected F2 recombinants within the QTL interval and backcrossed these mice to the parental strain B6J. A B6J x B6NJ F2 intercross (referred to as the Reduced Complexity Cross, RCC) contains approximately 32,000 genetic variants, instead of 4-35 million when using other inbred strains (Keane et al. 2011).
This project will focus on fine mapping and narrowing the QTL interval that our laboratory identified for Day 2 OXY-induced locomotor activity. First, a cross was created using closely related B6J and B6NJ mice. B6J and B6NJ mice were bred to create an F1 population. Non-sibling F1 offspring mice were then bred to make an F2 generation (RCC). Specific F2 offspring were then selected based on their distal chromosome 1 genotypes and backcrossed to B6J to generate offspring that were either homozygous B6J (J/J) or heterozygous (J/N) across distal chromosome 1.

We have decided to focus on narrowing the QTL interval for Day 2 OXY-induced locomotor activity. Because behavioral sensitivity is a Mendelian trait in the nearly isogenic background of the RCC, we assumed that only genotypes at distal chromosome 1 affected trait variation, and thus ignored genotypes across the rest of the genome. This allowed us to immediately begin fine mapping F2 recombinant mice, without the need for generations of backcrossing to first generate genome-wide isogenicity.

We hypothesize that the F2 recombinant mice will display the same phenotypic variation as the B6J/B6NJ parental strains as well as the RCC F2 mice, in which mice possessing one copy of the NJ allele show decrease locomotor activity under the influence of OXY. Furthermore, we hypothesize that we will fine map and narrow the QTL interval mediating this phenotype through the use of F2 recombinant mice.
METHODS

Drugs

Oxycodone hydrochloride (OXY, Sigma-Aldrich, St. Louis, MO USA) was dissolved in sterilized physiological saline (0.9%) prior to systemic intra-peritoneal (i.p.) injections. The OXY dose of 1.25 mg/kg was chosen based on preliminary studies on B6J and B6NJ (Figure 3).

Environment and Housing

All experiments were conducted in strict accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Boston University Institutional Animal Care and Use Committee. Colony rooms were maintained on a 12:12 hour light-dark cycle. Lights on at 0630 hours. Mice were housed in same-sex groups of two to five mice per standard shoebox-sized cage. Mice were fed standard laboratory chow and water available ad libitum except during testing. Mice were 50-100 days old at the time of testing.

Recombinant F2 Mice

Female C57BL/6J and male C57BL/6NJ mice were ordered from the Jackson Laboratory (Bar Harbor, ME, USA) at 7 weeks of age and were set up as F1 breeders. Non-sibling F1 offspring from these breeders were set up to breed B6J x B6NJ F2s. We chose to fine map RCC F2 offspring because of their nearly isogenic background. The B6J and B6NJ substrains differ at about only 10,000 SNPs across their genome (Simon et...
A B6JxB6NJ cross allows much of the genome to be eliminated when considering genes that underlie a QTL, an advantage other crosses utilizing different inbred strains do not have. We selected F₂ mice based on their specific genotypes at distal chromosome 1 and backcrossed them to B6J mice to generate offspring that were either homozygous B6J (J/J) or heterozygous (J/N) across various markers on distal chromosome 1 (160 Mb-186 Mb). Assuming that Day 2 OXY-induced locomotor activity is a Mendelian trait, only genotypes at distal chromosome 1 (163-190 Mb) were considered as potentially being associated with changes in behavior. Genotypes across the rest of the genome were disregarded.

To estimate sample size required to achieve 80% power (p < 0.05%) for fine mapping, we used the mean and standard deviations of OXY-induced locomotor activity based on genotype at the peak marker on chromosome 1 (rs51237371, J/J vs. J/N). Based on an effect size Cohen’s d = 0.79 (N = 59 J/J, N = 105 J/N; 206 F₂ mice total), we achieved 99.9% power to detect a significant difference (p < 0.05, two-tailed test). Based on this effect size, 27 homozygous J/J and 27 heterozygous J/N would be required to achieve 80% power (p < 0.05, two-tailed test). Therefore, we aimed for a sample size of 27 mice per F₂ recombinant strain.

**Behavioral Testing Apparatus**

The behavioral apparatus consisted of an open field (40 cm length x 20 cm width x 45 cm tall; Lafayette Instruments, Lafayette, IN, USA) that was used for locomotor studies surrounded by a sound-attenuating chamber (MedAssociates, St. Albans, VT,
USA). There were sixteen total apparatuses. For the experiments, the apparatus was partitioned into two equal sized compartments using an ion transparent plastic black divider containing a mouse entryway (5 cm x 6.25 m). This divider was flipped upside down during training to confine mice to one side of the testing apparatus. Two different floor textures were used on each side of the apparatus to differentiate the two sides (Plaskolite Inc., Columbus, OH, USA). Data from the apparatuses were recorded using a security camera system (Swann Communications, Melbourne, Australia). The videos were then subjected to video tracking analysis (Anymaze, Stoelting, Wood Dale, IL, USA). Video tracking allows to analysis of multiple variables that contribute to understanding drug-induced behaviors (Kirkpatrick and Bryant 2014) and the genetic components of these behaviors.

Behavioral Phenotyping

This project focused only on fine mapping Day 2 OXY-induced locomotor activity. An identical, yet truncated two day version of the place conditioned paradigm with 30 minute testing sessions was used (Figure 7). Mice were habituated in testing room for a minimum of one hour prior to start of experiment. Intra-peritoneal injections occurred immediately prior to mice being placed in the apparatus. On Day 1, mice were injected with saline (SAL, 10ml/kg) and allowed open access to both sides of the apparatus. On Day 2, mice were injected with OXY (1.25 mg/kg) and confined to the drug-paired side.
For this project, an abbreviated two day paradigm adapted from our laboratory’s previous studies utilizing a nine day place conditioning paradigm was used. Focusing only on Day 2 OXY-induced locomotor activity rather than withdrawal behavior (which would require a four-week protocol) allowed us to fine map the locus in a high throughput manner.

CPP/CPA = conditioned place preference, conditioned place aversion, SAL = saline
Behavioral Analysis

All behavioral analyses were run in R (https://www.r-project.org/) with a significance threshold set to p = 0.05. Primary outcome measures included locomotor activity on Day 1 and Day 2. Locomotor activity includes spins, rotations, and distance traveled. Recombinations were tracked at each SNP marker and families were analyzed based on genotypes at each marker. Families with similar genotypes were collapsed and analyzed together.

DNA Sequencing

DNA was isolated from mice tail snips using the salting out method. Using recently published sequencing results (Yalcin et al. 2011; Keane et al. 2011) (REL-1505), SNPs in the region of interest were determined and high confidence SNPs prioritized. DNA sequences for each SNP was obtained from the University of California, Santa Cruz Genome Browser (https://genome.ucsc.edu/). DNA sequences were inputted into the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to design primers for regions flanking the SNPs (at least 50 bp upstream and 20 bp downstream). Primers were ordered from Integrated DNA Technologies (Integrated DNA Technologies, Coralville, Iowa, USA) and used for polymerase chain reaction (PCR). DNA was diluted to 50 ng/µL for PCR reactions. PCR products were run on 1% agarose gel via electrophoresis. Successful PCR products (single, well-defined, bright band) were cut from a gel and extracted using QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Samples were sent to Genewiz (Genewiz, South Plainfield, NJ, USA) for Sanger sequencing. Sequences were viewed using ABI Sequence Scanner (Applied Biosystems, Inc., Foster
City, CA, USA). Following successful sequencing, custom Taqman SNP assays were designed (Life Technologies, Carlsbad, CA, USA). Chromosome 1 mice were genotyped at 7 SNPs (Table 1) within our region of interest with a mean distance of 4.25 cM (centimorgan) between markers. Genomic coordinates are based on mm10 (Build 38), and marker position (cM) was estimated using the sex-averaged position using the Mouse Map Converter (http://cgd.jax.org/mousemapconverter/) (Cox et al. 2009).
Table 1: SNP Assays Across Distal Chromosome 1

SNP markers used to monitor recombinations across distal chromosome 1, sorted by base pair/centimorgan position. B6J SNP serves as reference allele and is compared to B6NJ allele when determining where recombination events occurred.

cM = centimorgan, bp = basepair, SNP = single nucleotide polymorphism, B6J = C57BL/6J, B6NJ = C57BL6/NJ

<table>
<thead>
<tr>
<th>Chr</th>
<th>Position (bp)</th>
<th>cM</th>
<th>B6J</th>
<th>B6NJ</th>
<th>Type</th>
<th>dbSNP</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>163,132,699</td>
<td>70.6</td>
<td>A</td>
<td>T</td>
<td>SNP</td>
<td>rs6341208</td>
<td>Intergenic</td>
</tr>
<tr>
<td>1</td>
<td>167,677,640</td>
<td>74.9</td>
<td>T</td>
<td>C</td>
<td>SNP</td>
<td>rs49579753</td>
<td>Intergenic</td>
</tr>
<tr>
<td>1</td>
<td>172,612,184</td>
<td>80.0</td>
<td>G</td>
<td>A</td>
<td>SNP</td>
<td>rs255914894</td>
<td>Intergenic</td>
</tr>
<tr>
<td>1</td>
<td>181,318,003</td>
<td>84.6</td>
<td>A</td>
<td>C</td>
<td>SNP</td>
<td>rs51237371</td>
<td>Intergenic</td>
</tr>
<tr>
<td>1</td>
<td>182,984,657</td>
<td>86.1</td>
<td>C</td>
<td>A</td>
<td>SNP</td>
<td>rs48405612</td>
<td>Intergenic</td>
</tr>
<tr>
<td>1</td>
<td>187,620,470</td>
<td>91.8</td>
<td>C</td>
<td>T</td>
<td>SNP</td>
<td>rs212917841</td>
<td>Esrng</td>
</tr>
<tr>
<td>1</td>
<td>190,440,467</td>
<td>96.1</td>
<td>T</td>
<td>A</td>
<td>SNP</td>
<td>rs259427688</td>
<td>RP24-240P15.2</td>
</tr>
</tbody>
</table>
RESULTS

Replication of OXY QTL in F2 Recombinants

Our laboratory previously mapped a QTL to distal chromosome 1 that is responsible for mediating OXY-induced locomotor activity. To further fine map the QTL interval, F2 recombinant strains were created and tested in an abbreviated two day paradigm.

In examining the phenotypes of the F2 recombinants, there were no differences in locomotor activity on day 1 (SAL), regardless of genotype (Figure 8A). On day 2, mice that were homozygous B6J showed increased OXY-induced locomotor activity relative to mice that were heterozygous across distal chromosome 1 (Figures 8B). The findings are less clear for spins and rotations, however, mice that were heterozygous across distal chromosome 1 clearly show decreased activity on day 2 (OXY) compared to day 1 activity (SAL). These findings coincide with our laboratory’s initial study, in which parental strain B6J mice exhibited increased OXY-induced locomotor activity compared to parental strain B6NJ (Figure 3). They also coincide with our RCC findings, in which one copy of the NJ allele within the distal chromosome 1 locus was sufficient to decrease OXY-induced locomotor activity to a level similar to mice with two copies of the NJ allele (Figure 4).
Figure 8: Fine Mapping a QTL Mediating OXY-induced Locomotor Activity using Congenic Strains

(A) Day 1 total distance traveled. F₂ recombinant mice received SAL (i.p.).
(B) Day 2 total distance traveled. F₂ recombinant mice received OXY (1.25 mg/kg, i.p.).
(C) Three original F₂ strains used to backcross to parental B6J. Red dotted lines are locations of current SNP assays. Black areas are homozygous J/J. Grey areas are heterozygous J/N. White areas are unknown genotypes.

SAL = saline, OXY = oxycodone, B6J = C57BL/6J, SNP = single nucleotide polymorphism, i.p. = intra-peritoneal
Figure 9: OXY-Induced Locomotor Activity (Spins)

(A) Day 1 total spins. F2 recombinant mice received SAL (i.p.).
(B) Day 2 total spins. F2 recombinant mice received OXY (1.25 mg/kg, i.p.).

B = Homozygous B6J (J/J genotype), H = Heterozygous (J/N genotype), SAL = saline, OXY = oxycodone, i.p. = intra-peritoneal
Figure 10: OXY-Induced Locomotor Activity (Rotations)

(A) Day 1 total rotations. F₂ recombinant mice received SAL (i.p.).
(B) Day 2 total rotations. F₂ recombinant mice received OXY (1.25 mg/kg, i.p.).

B = Homozygous B6J (J/J genotype), H = Heterozygous (J/N genotype), SAL = saline, OXY = oxycodone, i.p. = intraperitoneal
Ruling Out the Region Distal to 187 Mb

The other goal of this project was to narrow down the QTL interval mediating OXY-induced locomotor activity on distal chromosome 1. The original QTL interval identified was 163 - 190 Mb with three working SNP assays in this region (give Mb position). Throughout the course of this project, we developed a total of 7 SNP assays within this region (table #). Furthermore, we are able to conclusively rule out the region distal to 187 Mb as containing the causal variants. We analyzed each congenic family based on their genotypes at each SNP assay. Families with similar recombination events were collapsed together and analyzed as a single line. Line 1A of Figure 11A represents this. Line 1A represents offspring from one of the original three F2 mice that was selected to backcross to parental strain B6J to create the F2 recombinant lines. Offspring were either homozygous J/J across the entire distal region of chromosome 1 (represented by black) or homozygous until 187 Mb, where they were then heterozygous J/N till the end of the chromosome. Because offspring from both these lines did not display differences in locomotor activity (Figure 11B), we conclude that this area of the chromosome does not contain the QTL of interest.
Figure 11: Rule out Region Distal to 187 Mb

(A) Line 1A represents the family analysis conducted to rule out the 187+ Mb distal region on chromosome 1 as containing the QTL. Families that were homozygous B6J (J/J) (black) were compared to families that were homozygous B6J except from 187-190 Mb, where they were heterozygous (J/N) (grey). White areas are unknown genotypes. Red dotted lines represent current SNP assays used to monitor recombination events.

(B) Locomotor activity on days 1/2 between lines compared. n = 53 (J/J), n = 67 (J/N)
Ruling out the Proximal 163-167 Mb Region of the QTL

Once we ruled out the distal region from 187 Mb to the end of the chromosome from consideration we collapsed data across additional families and focused solely on genotypes from 163 to 187 Mb (Line 1 becomes Line 1B, Figure 12A). Doing this, we were able to compare offspring that were homozygous across the region to offspring that were heterozygous at the proximal region of the chromosome (Figure 12A, Line 1B). Because there was no differences in OXY-induced locomotor activity between genotypes (Figure 12B), we conclude that the QTL responsible for OXY-induced locomotor activity is not located in any region proximal to 167 Mb on distal chromosome 1.
Figure 12: Rule out Region Distal to 163 Mb

(A) Lines 1/1B represent family analysis conducted to rule out proximal region of chromosome as containing QTL. After ruling out the distal 187+ Mb region, genotypes could be ignored from that region. This allows collapsing across families to analyze the proximal area (top line of Line 1 is collapsed with top line of 1B). Black areas are homozygous B6J (J/J), grey areas are heterozygous (J/N). White areas are unknown genotypes. Red dotted lines represent current SNP assays used to monitor recombination events.

(B) Locomotor activity on days 1/2 between lines compared. n = 9 (J/J), n = 24 (J/N)
DISCUSSION

This goal of this project was to fine map a quantitative trait locus responsible for opioid addiction-associated traits as measured via both acute behavioral sensitivity to the locomotor stimulant effect of OXY as well as spontaneous withdrawal following chronic OXY administration. OXY-induced locomotor activity on Day 2 was assessed, including distance traveled, spins, and rotations.

Previously, our laboratory identified a phenotypic difference in OXY behavioral sensitivity in two B6 substrains, B6J and B6NJ. Our laboratory then employed QTL mapping in the RCC of these two B6 substrains to identify a region on distal chromosome 1 that mediates OXY sensitivity (Figure 4). We selected F2 offspring based on their genotypes at distal chromosome 1 and then backcrossed these mice to the B6J parental strain, creating several congenic strains (referred to as F2 recombinant lines).

Our laboratory also found that the QTL interval for OXY sensitivity overlapped with a QTL for the emotional-affective component of OXY withdrawal, measured via change in time spent in the open arms of the elevated plus maze (Figure 5). The QTL intervals for these two phenotypes overlapped by approximately 10 Mb (171.0 – 180.5 Mb), therefore it is possible that one locus is responsible for the differences in behavior. The 95% Bayes interval for these QTLs is still quite large (D2 Distance: Chr1 171.0 – 181.3 Mb; Percent Open Arm Time: Chr1 151.7 – 180.5 Mb) and contains 83 SNPs (B6J vs. B6NJ) that could potentially be responsible for these phenotypes. However, because the RCC utilized the closely related C57BL/6J and C57BL/6NJ strains which differ by only approximately 10,000 SNPs across their genome (Simon et al. 2013), there are much
fewer genetic variants in the QTL interval compared to a typical F$_2$ cross. As a comparison, DBA/2J and C57BL/6J differ by approximately 4 million SNPs and 900,000 indels across their genomes (Keane et al. 2011).

Additionally, our laboratory used the limited genetic diversity of the RCC to implement a novel approach to fine mapping Mendelian traits such as OXY behavioral sensitivity. We selected F$_2$ mice with recombinations in the QTL interval, and backcrossed them while ignoring genotype across the rest of the genome. Because of the limited genetic diversity in the RCC and because we only identified a single locus accounting for most, of the genetic variance in OXY behavior, we reasoned that there would be minimal risk for epistatic interactions interfering with QTL detection following backcrossing and phenotyping of F$_2$ recombinant lines. This technique allowed us to replicate our QTL finding and define proximal and distal boundaries on the QTL.

While there is overlap between the QTL intervals for OXY sensitivity and withdrawal phenotypes, the question still remains as to whether one locus or two loci contribute to these phenotypes. The interval for OXY withdrawal extends to 151.7 Mb on its proximal end, which is approximately 20 Mb past the proximal end of the QTL for OXY sensitivity (171.0 Mb). One limitation of this study is the inability to test both phenotypes due to time constraints. The amount of time required to breed and phenotype 400 mice using a 4 week protocol (required for OXY-withdrawal) would have been too great. Focusing on day 2 OXY-induced locomotor activity allowed us to fine map the QTL in a high throughput manner. Thus, future studies are necessary to elucidate if there is a single QTL for OXY sensitivity and withdrawal or if there are two distinct loci.
While the distal end of chromosome 1 is a well-known QTL hotspot for neurobehavioral phenotypes (Mozhui et al. 2008), our laboratory has had trouble fine mapping the region of interest (171.0 – 180.3 Mb). One issue has been finding primer sets that successfully amplified a single gel band that was then successfully Sanger-sequenced for SNPs in the region. Our laboratory has designed and tested over forty primer sets for our region of interest, with the majority of sets failing when running PCR products on a 1% agarose gel or when PCR products were extracted from gel and sent in for Sanger sequencing. The primer set was considered failed when the gel gave multiple or undefined bands during imaging. A primer set was also considered failed when the Sanger sequencing results failed. Sanger sequencing results failed for a variety of reasons including SNPs sequencing: monomorphic, noisy with low confidence intervals for calls, or homomeric repeats before the SNP.

One theory that exists is that there may be a single master QTL on distal chromosome 1 that is responsible for several complex behavioral traits. Phenotypes related to motor behavior, escape latency, emotionality, seizure susceptibility, and responses to various substances have all been studied with crosses involving C57BL/6J and one of six other inbred strains (Mozhui et al. 2008). QTLs for each phenotype have mapped to distal chromosome 1, raising the question if there is a single master locus or a mixture of linked but functionally unrelated QTLs mediating the behaviors (Mozhui et al. 2008). One possible explanation is that there is a private SNP carried by B6J that none of the other inbred lines have, which is driving the different phenotypes.
The ultimate goal of any QTL mapping study is to find the QTG that underlies the QTL. While there has been advances in identifying QTLs for complex traits, moving from QTL to QTG remains the most critical, yet most challenging step. Criteria for QTG validation include relating gene function to QTL phenotype, identifying allelic polymorphisms, or assessing gene homology to determine if sequence is conserved across species (Tarantino and Eisener-Dorman 2012). Furthermore, ultimate QTG validation includes positional cloning and gene editing/replacement of the variant to demonstrate causality.

In conclusion, we replicated our QTL findings from previous the F2 study which indicated that a QTL on distal chromosome 1 contains genetic variant(s) underlying OXY-induced locomotor activity. F2 recombinant mice showed the same phenotypic variation as RCC and parental strain mice. Specifically, mice containing one copy of the NJ allele showed reduced OXY-induced locomotor activity. Furthermore, we were able to use the nearly isogenic background of F2 recombinant mice to immediately fine map the distal region of chromosome 1 by reducing both a portion of the proximal and distal interval from consideration. Based on family analysis, we conclude that the QTL lies in the region from 167 - 187 Mb.

Future Directions

Our QTL encompasses the QTL neurobehavioral hotspot (172-178 Mb) (Mozhui et al. 2008) and we are focusing on introducing additional recombination events within this interval to further narrow the locus. Future studies will include continuing to
monitor F₂ recombinant mice for recombinations within the QTL interval. Mice that possess such recombinations should be backcrossed to the B6J parental strain, in order to create additional F₂ recombinant lines to further narrow the interval. Once the QTL interval has been sufficiently narrowed, the same technique should be applied to fine map the QTL interval for OXY withdrawal, in order to determine if it is a single locus mediating both phenotypes, or if it is indeed two different loci responsible. Finally, examination of mRNA transcription, differential exon usage, and protein levels of multiple brain regions will allow us to further prioritize candidate genes and potentially candidate nucleotides that we will test directly via gene editing. Following the successful identification of a QTG mediating multiple opioid phenotypes, an important future direction will be to test for the genetic association of this gene in humans as well as the potential association with other addictions.
REFERENCES


Kumar, Vivek, Kyungin Kim, Chryshanthi Joseph, Saïd Kourrich, Seung-Hee Yoo, Hung Chung Huang, Martha H. Vitaterna, et al. 2013. “C57BL/6N Mutation in


