# OPIOID PATHWAYS IN METHAMPHETAMINE INTAKE

BY

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*A DISSERTATION*

*Presented to the Department of Behavioral Neuroscience*

*and the Oregon Health & Science University*

*School of Medicine*

*in Partial Fulfillment of the Requirements*

*for the Degree of Doctor of Philosophy*

**April 2015**

**OREGON HEALTH & SCIENCE UNIVERSITY**
**SCHOOL OF MEDICINE – GRADUATE STUDIES**

**School of Medicine**

**Oregon Health & Science University**

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

ADHD – attention deficit hyperactivity disorder

AMPA - a-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid

AMPH – amphetamine

BC - backcross

BUP – buprenorphine

B6 – C57BL6/J

Chr - chromosome

CNS – central nervous system

CPA – conditioned place aversion

CPP- conditioned place preference

CS+ - positive conditioned stimulus

CTA - conditioned taste aversion

DA – dopamine

DAT – dopamine transporter

DE – differentially expressed

D2 – DBA/2J

ERK – extracellular signal regulated kinase

ES – embryonic stem

FENT - fentanyl

GABA – y-aminobutyric acid

GAD – glutamic acid decarboxylase

GLU – glutamate

GWAS – genome wide association study

HCl – hydrochloride

HMACT - high methamphetamine activation

HSP – heat shock protein

IL - infralimbic

IP - intraperitoneal

IV – intravenous

LMACT – low methamphetamine activation

LOD – log of the odds

MA – methamphetamine

MADR – methamphetamine drinking

MAHDR – methamphetamine high drinking

MAHSENS – methamphetamine high sensitization

MALDR – methamphetamine low drinking

MALSENS – methamphetamine low sensitization

MOR - morphine

mPFC – medial prefrontal cortex

MOP-r – Mu-opioid receptor

NAc – nucleus accumbens

NC - nociceptin

NE - norepinephrine

NET – norepinephrine transporter

NIDA – National Institute on Drug Abuse

NMDA - N-methyl-D-aspartate

ORL-1 – opioid receptor like 1

PFC – prefrontal cortex

PKC – protein kinase C

PL - prelimbic

PPD - preprodynorphin

PPE – preproenkephalin

qPCR – quantitative polymerase chain reaction

QTL – quantitative trait locus

RI – recombinant inbred

RIST – recombinant inbred segregation test

SERT – serotonin transporter

SENS - sentization

SNc – substantia nigra pars compacta

SNP – single nucleotide polymorhphism

SNr – substantia nigra pars reticulata

SSRI – selective serotonin reuptake inhibitor

US – unconditioned stimulus

UTR – untranslated region

VMAT2 – vesicular monoamine transporter 2

VMb – ventral midbrain

VTA – ventral tegmental area

## ACKNOWLEDGEMENTS

There are a number of people that I would like to express my deepest gratitude to for influencing my decision to attend graduate school and to complete my doctoral research. First and foremost, I would like to thank my graduate school advisor, Dr. Tamara Phillips. Throughout the years she has taught me; to humbly accept criticism, how to critically think, and has molded me into a rigorous scientist. She has provided invaluable training in data analysis and statistical design. Without her, the last 6 years would not have been possible.

I also would like to recognize my undergraduate Professor, Dr. Lauren Liets, who influenced my interest in attending graduate school. During my undergraduate education at UC Davis, I took two courses from her; Advanced Systemic Physiology and Neurobiology of Addictive Drugs. The first course piqued my interest in research and the second introduced me to this field of research. I would also like to thank Dr. Ebenezer Yamoah, whom I worked with for 2 years prior to matriculating in this program. Dr. Yamoah discouraged me from applying to medical school and was ultimately the driving factor for me to apply to graduate school. During the two years that I worked in his lab, he provided indispensable training in molecular genetics and mouse colony maintenance. Dr. Yamoah has kept in close contact while I have been in graduate school and has been an excellent source for career advice.

I also would like to acknowledge the members of my dissertation committee, Drs. John Crabbe, Aaron Janowsky, and Mary Heinricher for their assistance designing and interpreting my dissertation research and constructive feedback on my dissertation document. Each played an integral role in specific components of this project and without a doubt strengthened my dissertation project and document.

I would also like to thank current and past members of the Phillips Lab (Carrie McKinnon, Sue Burkhart-Kasch, Harue Baba, Cheryl Reed, John Harkness, Noah Gubner, Angela Scibelli, Zeni Shabani, Jason Erk, Na Li, Bill Schutzer, and Raul Pastor) for providing a

collaborative work environment. In particular, I would like to thank Harue Baba for her attention to detail and her help to learn the microdissections used in this project and also some molecular genetic work that was not included in my dissertation project. I also would like to express gratitude to Carrie McKinnon, John Harkness, and Noah Gubner for assistance in some of the data collection. I also need to give credit to several lab members of the Janowsky lab that provided training for the molecular components of my project. Dr. Amy Eshleman has been instrumental in the binding assays and was very helpful with a [$^{3}$H]DAMGO autoradiography project that never came to fruition. In addition, Katherine Wolfram was instrumental in teaching me how to perform [$^{35}$S]GTPγS. Also, Robert Johnson was integral in teaching me to perform and analyze [$^{3}$H]DAMGO binding.

I also want to recognize the Ashworth Foundation which funded a trip to University of Pennsylvania to collaborate with Drs. Wade Berrettini and Glenn Doyle. Dr. Doyle taught me how to perform absolute qPCR and its analysis, as well as qPCR probe design, and molecular cloning. These projects were not included in my thesis but the training I received was invaluable.

I also want to acknowledge my classmates; Josh Kaplan, Amanda Barkley-Levenson, and John Harkness. I feel privileged to have worked beside them the past six years and look forward to watch their future careers unfold. Of course none of this would have been possible without the love and support of my parents (Ruth, Diane, and Ed), my in-laws (Bill and Sharon) and my fiancé, Kevin. Special thanks to my mother, who never once doubted me, appreciated the value of a graduate education, and has always encouraged me to pursue my dreams, no matter how bizarre. Kevin has been my rock and without his steadfast belief in me, I am positive I would have thrown in the towel long ago and I am looking forward to our next adventures together. The last six years has been a wild ride and I am thankful to have had so many friends and family members on the side lines cheering me on to complete my degree.

## ABSTRACT

Methamphetamine (MA) is a powerful psychostimulant and its excessive use is linked to neurotoxicity and neuropsychiatric disorders. However, not all initial users develop drug use disorders and it is possible that genetic differences render some individuals more susceptible to the addictive properties of MA compared to others. Genetic differences in avidity for MA have been studied using two replicate sets of selectively bred MA drinking (MADR) mouse lines that voluntarily consume either high (MAHDR) or low (MALDR) amounts of MA. Selective breeding alters allele frequencies; thus, alleles that increase MA drinking (MADR) have aggregated in the MAHDR line mice, whereas alleles that reduce MA intake have aggregated in the MALDR line mice. A gene mapping study identified a major effect genetic locus on mouse chromosome (Chr) 10 that accounts for more than 50% of the genetic variance associated with this differential MA intake. *Oprm1* lies within the mapped region and previous gene expression analysis added support for *Oprm1* as a candidate gene on Chr 10 that influences MA drinking. Based on published basic and human clinical data and preliminary data obtained within our laboratory, I hypothesized that *Oprm1* genetic variation and mu-opioid receptor (MOP-r)-regulated systems are important in influencing MA intake.

The first goal of this project was to examine potential differences in sensitivity to MOP-r-mediated effects in MADR mice. It was hypothesized that MOP-rs may be involved in the differences in MA intake between the MADR lines, and this might be reflected in a difference in MOP-r sensitivity and avidity. Sensitivity to the locomotor stimulant effects of the MOP-r agonist drugs morphine (MOR) and fentanyl (FENT) was measured and avidity for MOR was evaluated in a two-bottle choice MA drinking procedure. Sensitivity to the analgesic effects of MOP-r drugs was assessed using hot plate, tail flick, and the magnesium-sulfate-induced writhing test. In addition, MOP-r density and affinity were assessed between the MADR lines and also between C57BL/6J (B6) and DBA/2J (D2) strain mice, which were the founding strains for the selected lines. Opioid pharmacokinetics were also evaluated. No differences between the lines were

detected for sensitivity to the analgesic effects of MOP-r drugs, but MALDR mice had greater sensitivity to the locomotor stimulant effects of MOP-r agonist drugs, and consumed more MOR, compared to MAHDR mice. These data suggested that a negative genetic correlation exists between sensitivity to MOP-r agonist drugs and MA intake and also between MOP-r agonist intake and MA intake. In addition, MALDR mice had greater MOP-r density in the medial prefrontal cortex (mPFC), but not nucleus accumbens or ventral midbrain, compared to MAHDR mice. These data are consistent with the difference in *Oprm1* gene expression previously identified in the mPFC, but not the other two brain regions, and support my hypothesis that MOP-r-regulated effects may be involved in MA intake in MADR mice.

Based on differences in response to MOP-r drugs, the second goal of this proposal sought to examine the efficacy of MOP-r drugs to alter MA intake and drinking patterns. These studies administered either MOP-r agonist or antagonist drugs in a limited access two-bottle choice MA drinking procedure. It was hypothesized that MAHDR mice, which in comparison to MALDR mice, had lower expression of *Oprm1* in the mPFC, less MOP-r agonist-induced acute locomotor stimulation, and consumed less MOR, would more closely resemble MALDR mice for MA intake, when given a MOP-r agonist prior to MA drinking sessions. Some doses of the partial MOP-r agonist drug, buprenorphine, and the full agonist MOP-r drugs, MOR and FENT, reduced MA intake and altered drinking patterns in MAHDR mice. However MOR and FENT also reduced total volume consumed, suggesting that MOP-r agonist drugs may have induced a behavioral response that impeded drinking behavior. The MOP-r antagonist drug naltrexone did not alter MA intake. These data partially supported my hypothesis that MOP-r agonist drugs could alter MA intake and drinking patterns.

The final goals of this project were to verify the existence of the Chr 10 QTL for MA consumption using a more isogenic background and to gain better mapping resolution of the Chr 10 QTL. This aim was addressed using congenic strains of mice, which were created from B6 and D2 inbred mouse strains, the progenitor strains of the selected lines. One congenic

strain had a B6 segment that spanned Chr 10 0-7.72 Mb (the region between 7.58 and 7.72 Mb is of unknown genotype due to marker spacing and thus may or may not be of B6 origin), whereas the other had a B6 segment that spanned Chr 10 0-20.4 Mb (the region between 18.8 and 20.4 Mb is of unknown genotype due to marker spacing and thus may or may not be of B6 origin), both of which carried the B6 allele for *Oprm1*, located at Chr 10 6.75 Mb. Genotyping data used to detect the QTL for MA intake on Chr 10 had demonstrated that D2 alleles were associated with higher MA intake. I predicted that MA intake would be reduced in both congenic strains, compared to the D2 background strain. Contrary to my hypothesis, only one congenic strain (Chr 10 0-20.4 Mb) had decreased MA intake, compared to the D2 strain. These data indicate that genes proximal to the 7.58 Mb location on Chr 10 may be eliminated from consideration as quantitative trait genes influencing MA intake on Chr 10 and that a gene(s) that resides in the non-overlapping, up to 12.86 Mb segment (Chr 10 7.58-20.4 Mb) likely contributes to the genetic variation in MA intake between the MADR lines of mice. Though these data exclude *Oprm1* from consideration as a QTG, overall, the data support a genetic correlation between MOP-r density in the mPFC and MA consumption in the MADR lines of mice.

# CHAPTER 1: General Introduction

## Methamphetamine History and Abuse Epidemiology

Methamphetamine (MA) is a powerful central nervous system (CNS) stimulant, which may have detrimental health and social consequences when abused. Though currently considered an illicit substance, MA, or related drugs, has been prescribed as a nasal decongestant and to treat obesity, depression, narcolepsy, and occasionally attention deficit hyperactivity disorder (ADHD). The chemical structure of amphetamine (AMPH) was first determined in 1887 by Roman chemist Lazar Edelenau and the drug was named phenylisopropylamine. AMPH is comprised of a phenyl ring connected to a two-carbon side chain, with a methyl group at the carbon-1 position. Earlier that same year, Japanese chemist, Nagai Nagayoshi had isolated ephedrine from the Ma huang or *Ephedrine vulgaris* plant, which has been used as a traditional Chinese herbal medicine for nearly 5,100 years, primarily for its properties as a diaphoretic and circulatory stimulant (Chen K. K., 1927; Chen K. K. & Schmidt C. F., 1959). The chemical structure of AMPH was determined to be similar to ephedrine and produced similar physiological effects. In 1893, Japanese chemist Nagai Nagayoshi successfully produced MA, using ephedrine as a precursor.

In 1919, Japanese chemist Akira Ogata synthesized MA hydrochloride (HCl), also known as crystal meth, by reducing the ephedrine chemical structure with red phosphorus and iodine. MA is an N-methylated analog of AMPH and the presence of this additional methyl group increases the lipophilicity of the MA molecule, allowing the molecule to more freely cross the blood brain barrier. The addition of the methyl group also increases the chemical potency of MA (Schep L. J. *et al.*, 2010). The salt formulation of MA, made by the addition of a HCl group, increases drug lipophilicity and results in more rapid initiation of the physiological effects of MA. The addition of HCl to the MA structure also renders a solid state compound at room temperature, allowing the salt to be smoked. This is in contrast to MA isolated from ephedrine,

which is an oily liquid at room temperature conditions and can only be administrated via injection.

Both AMPH and MA induced alertness and were excellent bronchodilators, but it was not until the 1930s that Smith, Kline, and French patented the freebase, or pure basic form of AMPH, and sold it under the trade name of Benzedrine in an inhalant form to treat chest congestion (Rasmussen N., 2008). Its use increased during WWII, when fighter pilots (German, American, and Japanese) took AMPH in pill form to achieve greater alertness during combat. By the end of WWII, the rate at which physicians were prescribing AMPH had exploded, with uses from diet pills to antidepressants (Rasmussen N., 2008; Goodman S., 2010). MA use continued to skyrocket until the 1970s, when its addiction liability was fully recognized and Congress passed the Controlled Substance Act, which shifted AMPH to the Schedule II drug class. Currently, MA exists under the trade name Desoxyn®, but is rarely prescribed due to its abuse liability. AMPH, however, is still prescribed as Adderall®, to treat attention deficit hyperactivity disorder (ADHD), and is also used as a performance and cognitive enhancer (Poulin C., 2007). Methylphenidate, sold under the trade name Ritalin®, is a mixture of the active and inactive stereoisomer salts of MA, and is commonly prescribed to treat ADHD, as well as narcolepsy. Additionally, methylphenidate may be used to treat postural orthostatic tachycardia syndrome, during which a change in body position results in a large increase in heart rate and blood pressure.

**Methamphetamine Pharmacokinetics and Pharmacology**

MA is most commonly administered by smoking and via the intravenous (IV) route, as both cause MA to rapidly enter the bloodstream and cross the blood brain barrier, resulting in feelings of euphoria. According to a report from the National Institute on Drug Abuse (NIDA), drug users often prefer MA over AMPH because of its increased potency and duration of action. In Europe, AMPH use is more prevalent than MA use, and it is speculated that this might be due to a lack of chemical supply to manufacture MA. The pharmacokinetics of MA refers to its

absorption, distribution, metabolism, and excretion. Human subjects who received an IV bolus dose of [$^{11}$C] radiolabeled MA achieved peak drug levels within 9 min and cleared drug from brain tissue over a protracted period of 75 min (Volkow N. D. *et al.*, 2010). For reference, this is in contrast to cocaine, which has a proportionately greater accumulation of drug in brain tissue compared to MA and is cleared more rapidly from the brain (Riviere G. J. *et al.*, 1999). Riviere and colleagues speculated that the longer clearance time from brain may contribute to the known neurotoxic effects of MA. Further, in humans, the half-life of MA, measured in blood plasma, is approximately 12 h, which is significantly longer than the 1 h in rodents (Cook C. E. *et al.*, 1993; Riviere G. J. *et al.*, 1999). Because of this discrepancy in drug half-life, rodent animal models of MA binge and MA dependence often only model chronic drug activity for a fraction of the time that MA is active in humans.

MA is metabolized by the cytochrome P450 enzyme CYP2D6 into two major active metabolites: AMPH and 4-hydroxymethamphetamine. CYP2D6 metabolizes MA by oxidative deamination into AMPH or by one and two ring hydroxylation followed by methylation of one of the hydroxyl groups into 4-hydroxymethamphetamine (Santagati N. A. *et al.*, 2002). The hydroxyl-derivative metabolites are the primary ones detected in urine and in humans can be detected up to 7-8 days following MA ingestion, while un-metabolized MA may only be detected for 2-3 days following MA intake (Santagati N. A. *et al.*, 2002). AMPH is metabolized further into 3 major metabolites; 4-hydroxy AMPH by aromatic hydroxylation, norphedrine by β-hydroxylation, and benzyl methyl keoxamine by oxidative deamination. Of these metabolites, 4hydroxy-MA, AMPH, and 4hydroxy-AMPH are biologically active (Hendrickson H. *et al.*, 2006). Major MA metabolites, AMPH and 4hydroxy-MA, and major AMPH metabolite, 4hydroxy-AMPH, were compared in striatal and cerebellar tissue in rats for 120 min following [$^{11}$C] radiolabeled MA or AMPH administration. A comparison of similar doses of MA and AMPH, demonstrated that MA and AMPH share similar drug pharmacokinetics with approximately 60 min drug half-lives in brain tissue (Melega W. P. *et al.*, 1995). These data were comparable to a similar study

performed in humans, which showed an approximately 75 min half-life in brain tissue (Volkow N. D. *et al.*, 2010).

## Methamphetamine Mechanisms of Action

### Direct Actions of Methamphetamine

#### Monoamine Transporters

Once MA crosses the blood brain barrier and enters the CNS, it primarily targets monoamine transporters. The monoamine neurotransmitter, dopamine (DA) is involved in various processes including, movement, motivation, and reward, and DA re-uptake into the pre-synaptic DA neuron is regulated by the DA transporter (DAT) (Schultz W., 1997; Riddle E. L. *et al.*, 2005). Within DA neurons, the vesicular monoamine transporter-2 (VMAT2) packages DA into vesicles for its subsequent release. MA, AMPH, cocaine, and methylphenidate are substrates for the DAT and can bind to the transporter and inhibit its function. MA (and AMPH) can increase extracellular DA levels via two distinct mechanisms; by blocking monoamine transporters and preventing DA reuptake into the pre-synaptic terminal, and by increasing the release of DA into the extrasynaptic space via reverse transport at monoamine transporters. This second mechanism occurs via transporter-regulated entry of MA into the presynaptic terminal and interaction of MA with VMAT2. For example, with regard to DA, VMAT2 traffics vesicles and because MA is an amphipathic base it becomes sequestered into these acidic DA-containing vesicles. (Lee M. *et al.*, 2010). The basic pH of MA causes DA to become uncharged and follow its concentration gradient, until it is reverse transported into the synaptic cleft (Sulzer D. & Rayport S., 1990; Sulzer D. *et al.*, 1992). In comparison, cocaine only blocks monoamine transporters and does not cause reverse transport of DA.

In addition to DA, the norepinephrine (NE) transporter (NET) and serotonin (5-HT) transporter (SERT), play a significant role in the effects of MA and AMPH. Furthermore, the potencies of MA and AMPH to inhibit human and mouse monoamine transporters were compared and shown to shown to have similar sensitivities to each tested drug (Han D. D. & Gu

H. H., 2006). Though most pharmacological action of MA occurs at the DAT, the NET and SERT have been shown to down-regulate extracellular DA levels via DA uptake and thus, display some redundancy in function (Yamamoto B. K. & Novotney S., 1998; Sora I. *et al.*, 2001; Moron J. A. *et al.*, 2002).

DA neuron afferents project from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex (PFC) via the mesolimbic and mesocortical pathways. The mesocortical pathway refers to transmission of DA from the VTA to the cortex, while the mesolimbic pathway refers to transmission of DA from the VTA to the NAc (Leshner A. I. & Koob G. F., 1999; Le Moal M. & Koob G. F., 2007; Wise R. A., 2009). Increases in DA within the mesolimbic pathway are associated with feelings of reward during drug taking (Abraham W. C., 2008). However, other neurotransmitters play a role in drug-related effects. Fig. 1.1A is a schematic of the projections of DA, GABA, and glutamate neurotransmitters in the ventral midbrain (Vmb), which includes the VTA, NAc, and medial PFC (mPFC). These neurotransmitters modulate the mesolimbic DA system.

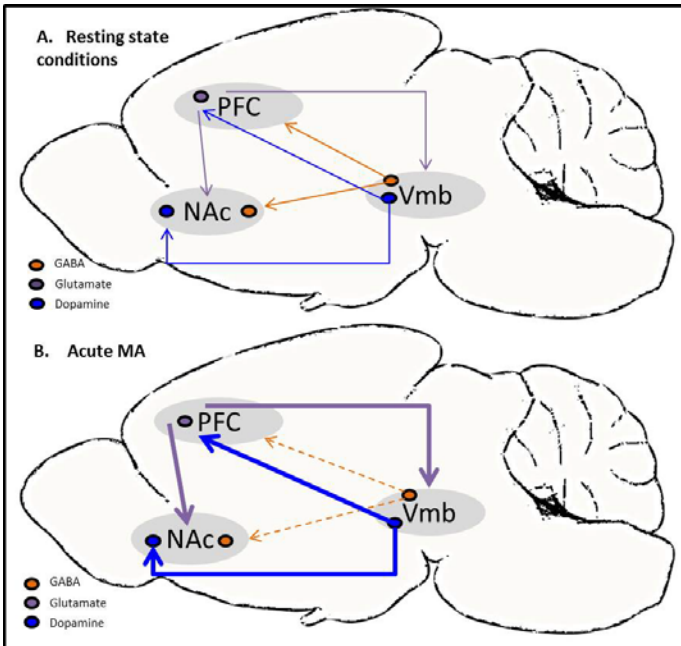


**Figure 1.1: Circuitry diagram depicting the predicted projections of GABA, glutamate, and dopamine in the Nac, Vmb, and mPFC regions.** Shown are (A) interactions during resting state conditions and (B) following acute MA administration. Arrows indicate the direction of projected neurotransmitter. Thicker lines indicate increased neurotransmitter activity and dotted lines indicate reduced neurotransmitter activity.

**Indirect actions of methamphetamine**

**y-Aminobutyric Acid (GABA) and glutamate (GLU)**

Indirect MA effects can occur as a consequence of direct actions on monoaminergic systems. GABA is the predominant inhibitory neurotransmitter in the CNS. There are two major receptor subtypes of the GABA receptor; the ionotropic $GABA_A$ receptor class and the metabotropic $GABA_B$ receptor class (Bormann J., 2000). GABAA receptors are chloride ion

channels and are sensitive to $GABA_A$ receptor antagonist, biccuculine. $GABA_B$ receptors are G-protein coupled receptors linked to potassium ion channels and are involved in mediation long-term potentiation, which is involved in learning and memory (Kaupmann K. *et al.*, 1997). The NAc is normally held under inhibitory control by GABA released from GABA interneurons. However, when activated, the NAc sends GABA projections to the substantia nigra and to the PFC via the medial dorsal thalamus. As portrayed in Fig. 1.1 B, during acute MA administration, GABAergic activity in the VTA is decreased, while extracellular levels of DA in the NAc are increased (Paulson P. E. & Robinson T. E., 1995). On the other hand, GLU is the most abundant excitatory neurotransmitter and is involved in cognitive processes such as learning and memory (Abraham W. C., 2008), and to play a role in drug-induced neuroplasticity (ref). GLU binds to metabotropic and ionotropic receptor subtypes. Metabotropic GLU receptors (mGLURs) are activated indirectly via G-protein coupled protein cascades. The metabotropic receptors comprise a class of 8 different receptor subtypes $mGLUR_{1-8}$. Ionotropic receptors form an ion channel pore that is activated when GLU binds to the receptor and is comprised of two main receptor subtypes of GLU receptors, N-methyl-D-aspartate (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA). NMDA receptors function to modulate post-synaptic activity by activating second messenger system cascades while AMPA receptors are responsible for most fast excitatory neurotransmission in the CNS and their modulation is thought to underlie synaptic plasticity (Pin J. P. & Duvoisin R., 1995). During MA administration, GABA neuron activity is altered within the substantia nigra by MA-induced DA release, resulting in disinhibition of thalamocortical projections and an increase in excitatory GLU release from the PFC, which projects to the NAc (Quintero G. C., 2013). AMPH has been shown to increase GLU extracellular levels in the cortex (Reid M. S. *et al.*, 1997), NAc (Reid M. S. *et al.*, 1997), VTA (Giorgetti M. *et al.*, 2001), and striatum (Del Arco A. *et al.*, 1999). These increases in GLU levels are driven by increased synaptic release of GLU as well as reduced tone on metabatropic GLU receptors 2 and 3 (mGluR2/3), which regulate the release of GLU (Moran M. M. *et al.*,

2005). The effects of MA on GLU projections from the PFC to NAc and VTA are shown in Fig. 1.B.

The GLU homeostasis hypothesis of addiction postulated by Kalivas describes an imbalance in crosstalk between cortical circuits that leads to a dysregulated reward system (Kalivas P. W., 2009). Kalivas proposed that during addiction to drugs, the balance between the limbic and motor circuits is disrupted and the motor circuit becomes overactive. This imbalance drives individuals to perform drug seeking behavior even when the motivational valence to do so is low. Specifically, the NAc functions as a gateway between the mesolimbic circuit, involved in motivation and drug reward, and the motor circuit, which is involved in active drug seeking behavior. Mechanistically speaking, the PFC manages the amount of GLU projected to the NAc and affects the strength of compulsive drug seeking behavior. During MA-induced homeostatic imbalance, basal extracellular levels of GLU are reduced, while the frequency of synaptic release is increased, and the rate of GLU elimination diminishes (McFarland K. *et al.*, 2003; Miguens M. *et al.*, 2008; Parsegian A. & See R. E., 2014). These hypotheses were demonstrated during an *in vivo* microdialysis study, where rats with previous IV self-administration of MA had decreased GLU levels in the dorsal medial PFC and NAc, and an increased efflux of GLU in these same regions during MA seeking (Parsegian A. & See R. E., 2014). These alterations in the balance of GLU, may support the transition from recreational drug use to drug dependence in human MA users, as drug seeking still occurs despite reduced reward during MA intake. However, these data are speculative and currently there is no evidence that demonstrates that this transition to drug dependence can be prevented by elevating basal GLU levels in the PFC or NAc during MA use, or by pharmacologically blocking increases in GLU in the PFC and NAc, during MA seeking.

**Opioids and Opioid Receptors**

The Opium poppy is historically one of the oldest used therapeutic drugs. Its use dates to 3400 BC in Mesopotamia by Sumerians who referred to it as the “joy plant” and it was used

by Hippocrates as a narcotic (c. 400 BC). During the 9th century, Opium was brought to China by the Arabs, but the first record of opium being used recreationally was not until 1493 by Xu Boling, who stated that opium was used “mainly to aid masculinity, strengthen sperm, and regain vigor.” Later during the 1700s, opium trade and opium dens became extremely prevalent (Brownstein M. J., 1993). Morphine (MOR), the active compound of opium, was isolated from the latex secreted from a scored poppy flower in 1804 and was commonly used for both diarrhea and analgesia (Macht D. I. *et al.*, 1915). Its potential for addiction liability substantially increased after the invention of the hypodermic needle in the 1850s (Kravetz R. E., 2005).

There are three major opioid receptor subtypes mu (μ), kappa (κ), and delta (δ), all of which are G-protein coupled and bind to endogenous opioid peptides (enkephalins, dynorphins, and ß-endorphins). Endogenous opioid peptides are present within the mesolimbic DA system and appear to be linked to positive reinforcing effects of drugs of abuse. However, the receptor subtypes appear to have differing roles. In general, activation of the μ-opioid receptor (MOP-r) is associated with analgesia and euphoria, activation of κ-opioid receptors has psychotomimetic effects, and activation of δ-opioid receptors induces seizure activity, particularly when higher doses of agonists are given (Pasternak G. W., 2001). The MOP-r is distributed across the brain, with a high density in neural regions responsible for sensory integration, such as nuclei supporting the olfactory, visual, nociceptive, and auditory systems. However, MOP-r expression is also found in the thalamus, amygdala, and midbrain regions, at a higher level in these regions than for the other opioid receptor subtypes. κ receptors are also expressed in sensory integration regions, suggesting involvement in sensory processing. δ receptors are found densely expressed in fewer regions, such as olfactory regions, the cortex, and caudate putamen (Mansour A. *et al.*, 1987).

MOP-rs co-localize on DA neurons and the increase in DA in the NAc and striatum induced by opioid receptor agonists is an indirect effect caused by disinhibition of GABAergic interneurons in the VTA and substantia nigra (Schad C. A. *et al.*, 2002). *in vitro* intracellular

recordings of either DA-containing cells or cells impinging on DA-containing cells in rat midbrain slices demonstrated that the opioid receptor agonist, Met-enkephalin, did not hyperpolarize DA-containing cells but did hyperpolarize secondary cells (Johnson S. W. & North R. A., 1992). There is also evidence that MOP-rs are expressed on GABA interneurons in the VTA and disinhibition of these VTA interneurons would silence the inhibitory GABAergic activity and increase DA neuron activity in the VTA, resulting in enhanced DA release in the NAc (Johnson S. W. & North R. A., 1992; Schad C. A. *et al.*, 2002).

The PFC sends descending GLU projections to DA containing neurons in the VTA (Sesack S. R. & Bunney B. S., 1989; Sesack S. R. *et al.*, 1989), and to GABA-containing neurons in the NAc (Carr D. B. & Sesack S. R., 2000b, 2000a). AMPH induces the release of endorphins (Olive M. F. *et al.*, 2001) in the NAc and application of the MOP-r agonist, D-Ala2-NMePhe4-Gly-ol-enkephalin (DAMGO) into the mPFC attenuated GLU-induced PFC neuron firing (Giacchino J. L. & Henriksen S. J., 1998). This effect was reversed upon application of the MOP-r specific antagonist, naloxone (Giacchino J. L. & Henriksen S. J., 1998). In addition, rodents administered MA acutely and following 7 daily injections had enhanced expression of preproenkephalin (PPE), the gene that encodes for enkephalin, in the striatum and enhanced preprodynorphin (PPD), the gene that encodes for dynorphin, in the striatum and NAc (Horner K. A. *et al.*, 2005; Tien L. T. *et al.*, 2007). In all, these data suggest that both MOP-rs and δ-opioid receptors engender several of the neurochemical effects of AMPH and MA but may play region-specific roles.

**Physiological and Subjective Effects of Methamphetamine**

Acute use of MA, like many other stimulant drugs, increases alertness, feelings of being content, and enhances levels of energy and stimulation (Hart C. L. *et al.*, 2008; Kirkpatrick M. G. *et al.*, 2012a; Kirkpatrick M. G. *et al.*, 2012b). These positive subjective ratings were dose-dependently correlated with improvements on a cognitive and psychomotor test battery (Hart C. L. *et al.*, 2008). However, MA intake may also be accompanied by negative subjective ratings of

irritability, racing heartbeat, and high levels of anxiety (Comer S. D. *et al.*, 2001). It is likely that there are individual differences in how MA is experienced.

Common reasons that an individual may receive medical attention during AMPH/MA intoxication are heart palpitations, hypertension, cardiac arrhythmias, and chest pain (Derlet R. W. *et al.*, 1989; Derlet R. W. *et al.*, 1990b, 1990a). In the peripheral nervous system, DA and NE regulate heart rate and blood pressure. Increased levels of these neurotransmitters induce increases in coronary vasoconstriction and calcium overload (Kaye S. *et al.*, 2007). Normally, an increase in heart rate would be accompanied by increased blood flow, vasodilation, and increased pulmonary response. However during MA intake, heart rate and blood pressure are increased, while blood vessels constrict, resulting in decreased amounts of cardiac oxygenation and an enhanced likelihood of having a myocardial infarction.

In addition to MA effects on the cardiovascular system, the constriction or dilation of blood vessels play a critical role in temperature regulation. The vessels control temperature by allowing heat to dissipate when blood vessels in the periphery increase their surface area or can prevent heat dissipation from occurring by reducing their surface area. Therefore, during MA use, when blood vessels constrict, thermoregulation does not occur efficiently. MA also increases metabolism in the CNS and in skeletal muscle, which increases body and brain temperature. Excessive body temperature may also induce the release of excitotoxic neurotransmitters (like GLU), and induce rhabdomyolysis, the breakdown of skeletal muscle proteins such as myoglobin; this can result in kidney damage (West P. L. *et al.*, 2010; Matsumoto R. R. *et al.*, 2014). Altering ambient temperature proportionately modulates the effect of MA on body temperature change (Bowyer J. F. *et al.*, 1994). Higher doses of MA (5 or 10 mg/kg) in a cool environment (18 ºC) induced hypothermia, but these same doses produced hyperthermia when administered in a warmer ambient environment (24 or 30 ºC) (Sabol K. E. *et al.*, 2013).

Chronic high doses of MA administered to rodents have been shown to cause damage to neostriatal DA fibers (Lorez H., 1981), and cause levels of striatal DA, DAT density and tyrosine hydroxylase (TH) activity to decrease (Fleckenstein A. E. *et al.*, 1997a; Fleckenstein A. E. *et al.*, 1997b; Fleckenstein A. E. *et al.*, 1997c). Decreases in striatal DAT have been linked to motor impairments and reduced performance on verbal learning and recall tasks and correlate with motor and memory deficits (Volkow N. D. *et al.*, 2001a). These neurochemical and behavioral alterations persist following a short, sustained abstinence from MA, but the neurochemical deficiencies recover following protracted MA abstinence (i.e., 12-17 mo) (Volkow N. D. *et al.*, 2001a; Thompson P. M. *et al.*, 2004). Therefore, long-term behavioral and cognitive effects may persist in the absence of neurochemical alterations. Mechanisms that result in this long-term damage are not well understood, but hyperthermia and the formation of reactive oxygen species are thought to contribute to neuronal damage that correlates with DA fiber damage (Thomas D. M. *et al.*, 2004).

In humans, neuroimaging studies have provided evidence that chronic MA use results in a decrease of DAT in the caudate and putamen that recovers over a protracted period of time (Volkow N. D. *et al.*, 2001a). Decreases in striatal DAT levels were associated with psychomotor impairments (Volkow N. D. *et al.*, 2001b). In AMPH-using subjects, MRI analysis demonstrated decreases in hippocampal volume and hypertrophy of brain white matter, which correlated with impaired hippocampal-based memory performance in a working recall task (Thompson P. M. *et al.*, 2004). Chronic MA use has been also affiliated with long-term behavioral effects including anxiety, confusion, insomnia, mood disturbances, and aggressive behavior. Some MA users may also develop psychotic symptoms that mirror those observed in schizophrenia. In particular, these features include auditory and visual hallucinations, paranoia, and delusions, which may persist for years after an individual has been MA abstinent (Sato M., 1992; McKetin R. *et al.*, 2006). That MA can induce psychosis with overlapping features of schizophrenia suggests a potential common underlying etiology for schizophrenia and MA-induced psychosis.

## Methods for Examining Rewarding and Aversive Effects of Drugs in Mice

No single animal behavioral assay can completely model every aspect of a human drug use disorder, however some sufficiently model relevant MA–induced behavioral effects. Rodent species are often utilized for drug research, because they have high genetic and physiological similarity to humans, are a mammalian species, are easy to handle, have rapid gestation periods, and have low maintenance costs compared to larger mammalian species, such as non-human primates. Mice were used in the current research, and described below are rodent models for measuring MA reward, aversion, and intake.

### Two-bottle Choice Drinking

Voluntary two-bottle choice drinking provides a method to measure the amount of drug consumed and preference for the drug-containing solution, and models human, free-choice drug intake. Oral ethanol intake best translates between humans and rodent models, as it is the most common route of ethanol use in humans. However, other drugs of abuse are also consumed orally in humans. Humans commonly smoke the crystalline form of MA or inject a liquid form IV; however, in some cases, MA is self-administered in pill form. For example, the prescription drug, MA HCl (Desoxyn®), is an oral preparation that is currently prescribed to treat obesity, narcolepsy, and ADHD. The ability to assess individual differences is a hallmark of two-bottle choice procedures, in which one bottle containing drug dissolved in water is offered along with a second bottle containing only water. The bottles may be offered to animals with open access (all day) or for limited time periods during the day, depending, in part, on the goal of the research (Belknap J. K. *et al.*, 1993a; Le A. D. *et al.*, 1994; Becker H. C. & Lopez M. F., 2004; Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011).

Use of a lickometer apparatus provides better temporal resolution than, for example, hourly measures of volume consumed. In addition, pattern of drinking behavior can be assessed. The lickometer device is essentially an open electrical circuit that consists of a Plexiglas chamber with a stainless steel wire floor and metal sipper tubes inserted into the

portholes of the chamber. When a mouse simultaneously stands on the metal floor and makes contact with a metal sipper tube, the circuit is closed and a cumulative lick record is generated for each tube. Licks over time and total volume consumed can be recorded, and bout patterns can be generated, for example, based on a series of a defined number of licks performed within an *a priori* time interval (Ford M. M. *et al.*, 2005; Ford M. M. *et al.*, 2009; Pastor R. *et al.*, 2010; Dwyer D. M. *et al.*, 2011). Use of a computer program allows several additional variables to be compiled, including total sipper contacts (licks), number of bouts, size of bouts (licks), duration of bouts (sec), interbout interval, bout lick rate (licks/min), and latency to first bout (sec).

The use of a lickometer system is an effective way to evaluate the impact of pharmacological treatments on oral intake (Frisina P. G. & Sclafani A., 2002). For example, pretreatment with $GABA_A$ receptor agonist drug, gabaxadol HCl [THIP], significantly reduced ethanol drinking during the first 5 h of a 24h session, attenuated first bout size, and increased the latency to first ethanol bout (Ramaker M. J. *et al.*, 2011). In cases where a drug effect is short-lived, use of a lickometer is more likely to allow such transient drug effects to be detected. Animals may initially show a drug effect and then increase their drug consumption later in the session, resulting in what would be detected as no effect in a typical study using a single volumetric recording over a more protracted period of time. Further, this procedure can be validated by correlating the intake volume to the number of licks that occur during each session. The lickometer device has been used most prevalently in the ethanol field (Boyle A. E. *et al.*, 1992, 1997; Samson H. H., 2000), though drinking microstructure of other fluids, including water, sucrose, polycose, sweetened milk, and lecithin, has been examined (Schneider L. H. *et al.*, 1990; Davis J. D. *et al.*, 1999; Johnson A. W. *et al.*, 2010).

A few things should be noted. Because not all animals consume the same amount of drug in two-bottle drinking procedures, the amount of drug exposure across animals cannot be controlled. In addition, it may be difficult to determine the source of the motivation of an animal to consume the drug-containing solution, as factors such as thirst and taste may have an

influence, in addition to reward and aversion (Tabakoff B. & Hoffman P. L., 2000). Hunger may also influence drug consumption, particularly for a drug like ethanol, which has caloric value. However, MA has no caloric value, and human subjects given oral MA actually had decreased daily total caloric intake compared to subjects given a placebo (Comer S. D. *et al.*, 2001; Hart C. L. *et al.*, 2001). Other behavioral assays to determine whether the drug is perceived as rewarding or aversive can be conducted and are discussed in depth below (Leeman R. F. *et al.*, 2010).

**Operant Models**

Models of operant conditioning use positive reinforcement to shape the behavior of a subject and model the reinforcing efficacy of a drug. In a typical operant procedure, subjects are trained to perform a behavior (such as lever pressing or nose poking) and receive a food or drug reward for their work. Depending on the drug reward, animals may be working for an oral (e.g. dipper full of ethanol), IV or intracranial infusion of drug. Under some conditions, these procedures allow the motivation of an animal to obtain the reward to be assessed and dissected from consummatory behavior (Tabakoff B. & Hoffman P. L., 2000). The amount of work the animal is willing to perform to receive access to the drug reward indicates that the drug being self-administered is functioning as a positive reinforcer (Weiss F. & Koob G.F., 1991; Tabakoff B. & Hoffman P. L., 2000).

Operant models may use several schedules of reinforcement. Fixed ratio schedules do not provide information about the relative reinforcing effects of drugs and are reported as responses/second. Fixed interval second order schedules examine how drug-associated stimuli maintain the responding on a task that is ultimately reinforced by the delivery of a drug (Panlilio L. V. & Goldberg S. R., 2007). These schedules are often quite complicated and ability of a reward to maintain schedule-appropriate rates of response suggests that the reward is reinforcing (Goldberg S. R. & Kelleher R. T., 1976; Banks M. L. & Negus S. S., 2012). Progressive ratio schedules directly assess the effectiveness of a reinforcer by requiring an

increasing number of responses for each reinforcer to be delivered, until no responding occurs, which is called the break point (Panlilio L. V. & Goldberg S. R., 2007; Banks M. L. & Negus S. S., 2012). If a drug is reinforcing, then the animal should continue to respond under increasing work requirements. Fixed ratio schedules are often favored over more complex schedules such as progressive ratio because of their straight-forward nature, where a fixed number of responses results in the delivery of a reinforcer (Richardson N. R. & Roberts D. C., 1996).

Most operant procedures include both an 'active' and 'inactive' manipulandum that allows general responding to be assessed. The inclusion of an inactive lever controls for high responding that is not contingent on receiving the reward and may provide information about whether lever-pressing activity is induced by the drug (Pickens R. & Thompson T., 1968). However, operant procedures are limited by the amount of training time required. Most often, investigators have examined IV self-administration in rats rather than mice, because the diameter of their veins is larger, and thus the surgery is more successful and inserted cannula are less likely to become occluded or atrophy. Although IV self-administration has been established in mice, it is much difficult to construct, implant, recatheterize, and maintain catheter patency compared to rats (Thomsen M. & Caine S. B., 2005), and is not suitable when large numbers of mice must be tested.

**Conditioned Place Preference and Conditioned Place Aversion**

A model used to assess sensitivity to the conditioned rewarding effects of a drug is the place conditioning procedure. Though there is a vast preclinical literature examining drug conditioning in animals, there are also data in humans receiving oral MA, showing significant preference for drug-associated physical and virtual environmental cues, which correlated with the degree of drug liking (Childs E. & de Wit H., 2009; Mayo L. M. *et al.*, 2013). This procedure is based on classical Pavlovian conditioned approach, where the learned association of the effects of a drug is made with an environmental cue, such as a light, cage floor texture, location, or smell (Cunningham C. L. *et al.*, 2006). Here, the environmental cue or spatial location is the

conditioned positive stimulus (CS+) and the effect of the drug treatment is the unconditioned stimulus (US). The ultimate goal of the procedure is to transfer the US to the CS through a learned association, so that the motivational valence of the US can be assessed.

After completing the conditioning phase of the CPP procedure, a common method for assessing drug-associated cue preference is to generate an unbiased difference score between time spent in association with the drug-paired cue on the test day and the time spent in association with the same cue during a pre-conditioning preference test. Preference in a CPP procedure is most commonly measured when the animal is in a drug-free state, but can also be measured after drug administration. Generally, the goal is to assess whether the animal prefers to spend time in the drug-associated or placebo-associated context. By administering the preference test in a drug absent state, the confound of a drug effect on locomotor activity is removed. However, testing in a drug-present state can get at state-dependent conditioned effects. For example, in mice that were conditioned to one cue with MOR and another with saline, a marked increase in the magnitude of MOR-CPP was seen in the drug-present test, compared to the drug-absent test (Bespalov A. Y. *et al.*, 1999). This study also examined the effects of other MOP-r drugs on the expression of CPP and demonstrated that CPP was expressed only after MOR treatment and not after treatment with other MOP-r agonist drugs, in mice that had been conditioned with MOR. These data are consistent with the concept of state-dependent learning retrieval, such that only MOR produced the expression of CPP (Overton D. A. & Batta S. K., 1979), but also suggest that there are differences in the subjective effect of MOR vs. other MOP-r agonist drugs.

Strengths of CPP include that the procedure is high-throughput given adequate equipment, subjects can be tested in a drug-absent or drug-present state, it is adaptable to many species, it allows locomotor activity to be simultaneously recorded, and both the development of CPP, when the initial training occurs, and the expression of CPP can be separately examined and pharmacologically manipulated (Bardo M. T. & Bevins R. A., 2000;

Cunningham C. L. *et al.*, 2006). MA and AMPH have been shown to induce a robust CPP response, suggesting that these drugs can produce strong conditioned reward (Pickens R. W. & Crowder W. F., 1967; Yokel R. A. & Wise R. A., 1976).

Potential interpretational difficulties with CPP include biases in initial cue preference, which could interfere with the formation of a drug-induced cue preference. This issue may be addressed by performing a drug-free pre-test to determine if animals spend a greater proportion of time in association with one cue over the other before the drug has been paired with either cue (Cunningham C. L. *et al.*, 2003). Another potential issue is that the animals receive the drug passively, rather than actively self-administering it, and subjective effects may be different under these conditions, due to different physiological effects. For example, it has been shown that a DAergic response that occurs in the NAc of AMPH-IV self-administering rats, is not seen in paired yoked control rats that passively receive the identical amount of AMPH (Di Ciano P. *et al.*, 1998).

It is also possible to obtain data suggesting that a drug has aversive effects, using the same conditioning procedure; in this case, the outcome would be conditioned place aversion or CPA (e.g., Shabani et al., 2011). If the animals spends less time on the drug-paired side of the chamber compared to either the pre-test preference or time on the non-drug-paired side, this is interpreted to indicate that the animal finds the administered drug aversive. More robust CPA may be seen when the drug is given after, rather than prior, to cue exposure, and opposite effects may even be seen. For example, when AMPH was given before CS exposure, robust AMPH-induced CPP was observed, but when AMPH was given after CS exposure, AMPH-induced CPA was observed (Fudala P. J. & Iwamoto E. T., 1990). Whether CPA was seen when MA is given after CS exposure was genotype-dependent in one study (Shabani S. *et al.*, 2012b). These time-dependent effects seem paradoxical. A potential explanation for CPA when the drug is given after cue exposure is that drug administration produces a short duration aversive effect that is followed by a longer rewarding effect, and thus, that the short-term

aversive effect is most closely associated with the cue in this procedure (Cunningham C. L. *et al.*, 1997).

**Conditioned Taste Aversion**

Rapid associations between biologically relevant stimuli allow potentially harmful substances to be avoided. Conditioned taste aversion (CTA) may be used to assess aversive effects of a drug in a conditioning procedure that is not dependent on context; rather, it uses a novel taste. CTA is the learned association between a novel taste, such as that of a sodium chloride or saccharin solution, and a paired drug, which may induce nausea or negative subjective effects. Before initiating the taste conditioning procedure, animals are adapted to having restricted access to water to motivate drinking behavior during the time when the novel flavor is paired with the drug effect (Davis C. M. & Riley A. L., 2010; Riley A. L., 2011). If the drug causes an aversive experience, then less of the novel solution should be consumed when subsequently offered (Cappell H. & LeBlanc A. E., 1971). If negative subjective effects of the paired drug are not experience, then the subject will continue to consume pre-drug pairing levels of the novel solution (e.g., Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011).

Strengths of the CTA procedure include that the methods are straightforward and that CTA can be produced by an array of compounds, which vary from emetic compounds to psychoactive drugs (Hunt T. & Amit Z., 1987). Furthermore, the association between the novel taste and paired drug can occur extremely rapidly (aversion may be seen following one pairing), and this association is learned over long-time delays, allowing for appropriate associations between biologically relevant stimuli (such as taste and sickness, rather than audiovisual cue and sickness) (Verendeev A. & Riley A. L., 2012). AMPH has been shown to induce CTA to saccharin following a single systemic injection; saccharin is a highly preferred substance to rodents (Cappell H. & LeBlanc A. E., 1971). The range of AMPH doses that induced CTA in that study (5-40 mg/kg) was within the range that is self-administered by rodents and was below the range of doses that would produce AMPH-toxicity (Cappell H. & LeBlanc A. E., 1977).

Weaknesses of the CTA procedure include difficulties in interpretation of the data when the same dose used to establish CTA can also independently produce CPP. Drugs that are self-administered are not simple pharmacological agents and have multiple stimulus effects, not all of which may be positive reinforcing effects (Lynch W. J. & Carroll M. E., 2001; Le Moal M. & Koob G. F., 2007; Riley A. L., 2011). These apparently opposite effects may seem paradoxical and are interpreted as a compound effect of a single dose of drug, which has both positive and negative elements (Wise R. A. *et al.*, 1976; Wang Y. C. *et al.*, 2010; Riley A. L., 2011; Verendeev A. & Riley A. L., 2011).

**Methods for the Investigation of Genetic Influences on Drug Effects**

Many of the advances in identifying the underlying genetic contributions to addiction stem from the use of genetic animal models. Specifically, mice have been extensively used in forward genetics approaches to study the genetic basis of complex addiction-related traits. A complex trait is a phenotype that may be influenced by multiple genes, multiple environmental factors and gene x gene as well as gene x environment interactions. The genetic basis of variation in a complex trait is challenging to identify, in comparison to a Mendelian trait, which is controlled by a single gene. The forward genetics approach begins with individual differences in the level of a drug-altered behavior, which are thought to have genetic variation as at least part of their source and may utilize recombinant inbred mouse strains (and other specialized breeding populations like advanced intercross lines), quantitative trait locus (QTL) analysis, and selectively bred lines, as well as global gene expression analyses. This approach then works backward to identify the gene(s) underlying the phenotypic variation. On the other hand, a reverse genetics approach examines the effect of a change in genetic sequence on a phenotype. Reverse genetic approaches used to examine the involvement of a gene of interest include single gene knockout mice, RNA interference, and single gene expression analyses. Several of these genetic strategies and their strengths and weaknesses are discussed below.

**Quantitative Trait Locus Mapping**

QTL mapping allows a trait of interest to be mapped to specific chromosomal regions, indicating that genetic variation in those locations plays a role in trait variation. A mapping population, which could consist of a pair of selected lines, a heterogeneous population derived by intercrossing 2 or more inbred strains or more complex recombinant inbred strains, is tested for a phenotype of interest and genotyped genome-wide (Lebowitz R. J. *et al.*, 1987). Microsatellite markers can be used to detect polymorphisms at intervals along each chromosome, and marker identity (e.g., if all alleles come from the B6 and D2 strains, then whether the allele is of the B6 or D2 form) is correlated with trait magnitude. A correlation of a size that meets a stringent significance criterion that accounts for multiple comparisons (Belknap J. K., 1992; Neumann P. E., 1992; Lander E. S. & Schork N. J., 1994; Belknap J. K. *et al.*, 1996) indicates that a QTL exists in that chromosomal location (Bergeson S. E. *et al.*, 2001). Furthermore, the strength of the evidence for a QTL can be statistically examined using a logarithim (base 10) of the odds of genetic linkage or a LOD score, and the confidence interval can be determined, in part, by considering whether marker-trait correlations are significant for several markers in the same general chromosomal region.

QTLs mapped in populations with larger regions of linkage disequilibrium will have poor resolution, and could have a confidence interval of 20 centimorgans (cM) or even larger, which contains around 1,000 genes, based on the assumption that 1 cM contains about 50 genes (Belknap J. K. *et al.*, 2001). Mapping resolution is contingent on the type of mapping population used (e.g., F2 vs advanced intercross lines) and the density of marker coverage. The ultimate goal is to map the QTL to a 1 cM or less interval, so that fewer genes can be studied as possible quantitative trait genes (QTG). To move from the QTL interval to examining the potential impact of one or more genes in the region that influences the magnitude of the trait, gene expression, pharmacological and gene-specific manipulations may be performed. In humans, the analogous genetic strategy to map a trait to a particular genetic location is the genome-wide association study (GWAS), in which large sample sizes of subjects must be

included to gain enough power to reach significance. Another technique includes the SNP array, which assesses SNP variants genome-wide to determine whether a certain gene variant is associated with a trait or disease state.

QTL and GWAS mapping has been used in a variety of addiction-related projects and to map quantitative traits to QTL regions and identify candidate genes. For example, in a previously described MA-related selective breeding project, a QTL analysis in the high MA activation (HMACT) and low MA activation (LMACT) lines mapped a QTL to mouse Chr 15 (Kamens H. M. *et al.*, 2005). Gene expression profiling (approach described below) using tissue from the NAc of drug-naïve mice from these lines identified a 10-fold difference in expression of casein kinase 1 epsilon, *Csnk1e,* located on Chr 15, (Palmer A. A. *et al.*, 2005). Peripheral administration, as well as microinjection into the NAc, of PF-670462, a selective inhibitor of the *Csnk1e* gene product*,* attenuated the MA-induced increase in locomotor activity, confirming that *Csnk1e* is critical to the stimulant response (Bryant C. D. *et al.*, 2009b). When these findings were carried forward to humans, a non-coding SNP in the *CSNK1E* gene was found to be associated with subjective response to AMPH (Veenstra-VanderWeele J. *et al.*, 2006).

Strengths of QTL analysis include that there is high statistical power even when few markers are genotyped, such that small effect QTLs can be mapped. Suitable populations for finer QTL mapping include interval specific congenic strains, and populations with a high density of recombinations, such as advanced intercross lines. A high rate of recombination decreases linkage disequilibrium and increases the number of genetic markers that need to be examined (Palmer A. A. & Phillips T. J., 2002). However, QTL analysis can only capture the genetic variation present within the mapping population, thus relevant genetic influences may be missed (Grisel J. E., 2000). Using next generation sequencing technology and considerable existing knowledge of allelic diversity in multiple species, such issues have been addressed.

A major weakness of QTL analysis has been the difficulty involved in identifying the gene(s) influencing the QTL and that very few QTGs have actually been identified compared to

the large number of QTLs that have been mapped (Mackay T. F., 2001a). Buck and colleagues successfully moved from QTL to QTG for a QTL that was detected on mouse Chr 4 for withdrawal-induced convulsions following acute and chronic ethanol exposure (Buck K. J. *et al.*, 1997). Use of congenic strains reduced the QTL interval to a 1.8 Mb region and identified the multi-PDZ gene, *Mpdz*, as a candidate QTG (Shirley R. L. *et al.*, 2004).

**Standard Inbred and Recombinant Inbred Strains**

Standard inbred mice are created by at least 20 consecutive generations of brother by sister matings, resulting in each individual of a given strain being virtually genetically identical and homozygous at every locus (Staats J., 1985; Casellas J., 2011). Panels of inbred strains of mice have been often used to examine genetic correlations and to estimate trait heritability (Crabbe J. C. *et al.*, 1990; Falconer Douglas S. & Mackay Trudy F. C. , 1996; Mogil J. S. *et al.*, 1999a; Rhodes J. S. *et al.*, 2007). A genetic correlation refers to the extent to which a common set of genes can be said to influence two different traits and can be determined using inbred strain means. Information regarding polymorphisms and sequence among inbred strains has been collected and is available publically in databases, such as the Mouse Phenome Database, which contains collaborative information from multiple investigators for an array of behavioral, physiological, genotypic, and morphological traits from several inbred mouse strains (Paigen K. & Eppig J. T., 2000). This information has reduced redundancy in research and facilitated genetic mapping (Belknap J. K., 1998).

Typical genetic mapping studies create a heterogeneous mapping population by cross-breeding 2 or more inbred strains. Each of the resulting offspring is genetically unique with a mosaic pattern of crossing- over events. Because each individual is genetically unique, each must be genotyped and phenotyped, and therefore a relatively large number of individuals are required for genetic analyses, and the number affects statistical power in genetic mapping studies for complex traits (Chesler E. J. *et al.*, 2001; Darvasi A., 2001). Advanced intercross lines are created by additional generations of intercrossing (e.g., F10) so that genetic diversity is

increased and more recombination events occur (Palmer A. A. & Phillips T. J., 2002). Most recently, the diversity outcross and collaborative cross heterogeneous stock mouse populations have been recommended for use because of improved genetic diversity and mapping precision due to an increased frequency of recombination events (Chesler E. J., 2014). The enhanced number of recombinations allows for QTL mapping to a small interval to be accomplished, with a reduced number of individuals.

Recombinant inbred (RI) panels of mice are created by inbreeding pairs of mice to homozygosity, starting with recombinant offspring from inbred strain crosses. A panel of RI strains consists of a set of mosaic genomes from the original inbred strains. For example, the BXD panel of RI strains was derived from the recombinant offspring of the F2 cross of the B6 and D2 inbred strains, and has been used in many mapping studies for complex behavioral and physiological traits. In addition, they can be used to study genetic correlations. For MA, a panel of 25 BXD RI strains was used to determine the genetic correlation between MA-induced body temperature change, locomotor activity response, and stereotypy. The largest genetic correlation existed between MA-induced body temperature change and home cage locomotor activity (Grisel J. E. *et al.*, 1997). Further, because RI data are stored in collaborative databases, several decades of behavioral data can be re-examined and genetic correlations can be calculated between behavioral and gene expression variation, using gene expression data collected in brain tissue from the identical RI panels (Chesler E. J. *et al.*, 2003). This type of analysis generates hypotheses regarding QTL candidate genes using a systematic approach to examine the variation in gene expression for genes that reside in an identified behavioral QTL region.

Panels of RI mouse strains provide all of the same advantages as standard inbred mouse strains, including their reproducibility and the ability to integrate historical data. Additional strengths of the use of RI panels include that once the genetic identity at several chromosomal markers have been determined for one member within each RI strain, additional offspring do not

need to be genotyped and the focus of subsequent work can be spent on phenotyping, rather than genotyping (Bailey D. W., 1971).Though there are many advantages to using this type of genetic animal model, a disadvantage for genetic mapping is that most RI panels are derived from 2 parental inbred strains, limiting genetic diversity. Low genetic diversity also arises from a large overlapping proportion of the genome between most inbred mouse strains, such that a non-uniform distribution of genetic variation across the genome in the RI panel may occur (Roberts A. *et al.*, 2007; Yang H. *et al.*, 2007). Most RI panels consist of as few as 15-35 individual strains from an intercross of two different inbred mouse strains, which limits the power and precision of statistical methods used to map QTL (Plomin R. *et al.*, 1991a; Plomin R. *et al.*, 1991b). However, though some of the original BXD RI strains have been retired, a total of 102 strains have been fully inbred and 81 of these are now available for commercial use (www.genenetwork.org).

RI strains tap into the naturally occurring recombination events that occur during meiosis, however, the use of advanced intercross strategies have allowed the number of recombination events to increase from 48.1 recombinations per strain in the older BXD strains to 82.4 recombinations per strain, in the more recently derived strains (Pierce A. J. & Jasin M., 2005; Shifman S. *et al.*, 2006). In comparison to RI strains starting from the F2 cross, advanced intercross strategies perform repeated crossing for 9-14 generations before initiating inbreeding, allowing nearly twice as many recombination events to occur (Pierce A. J. & Jasin M., 2005). However, an even more genetically diverse RI strain panel, known as the Collaborative Cross, with 1,000 strains, was derived from an 8-way cross of inbred mouse strains and offers greater genetic mapping power, the ability to detect epistatic effects, and increases in mapping resolution (Vogel G., 2003; Threadgill D. W. *et al.*, 2011). Epistasis refers the effect of one gene being dependent on the presence of one or more other genes in the genetic background that acts as a modifier gene. Because of the large number of genetic markers that must be evaluated by genotyping for a genome-wide analysis, there is a high rate of Type I statistical

error, or the rate of false positive findings. Thus, the analysis of RI data must include an adjustment of the threshold (reduced p value) for concluding that a finding is statistically significant (Belknap J. K., 1992).

**Single Gene Manipulations**

Single gene knockout animals follow a reverse genetic approach, where a candidate gene is manipulated by mutation and its contribution to a trait of interest is evaluated. To create a targeted mutation, mouse embryonic stem (ES) cells are collected and a vector containing the targeted gene mutation is introduced into the ES cells by either electroporation or microinjection, and the mutated gene can be incorporated into the DNA of the ES cell by homologous recombination (Nelson R. J. & Young K. A., 1998). The ES cells are then injected into blastocysts of mouse embryos and later implanted into psuedopregnant female mice, where the embryos are carried until the impregnated dam gives birth to the chimeric offspring (Sedivy J. M. & Sharp P. A., 1989; Soriano P., 1995; Gerlai R., 2001). The chimeras are heterozygous null mutant offspring and in the most rigorous approach are used as breeders to generate offspring that must be genotyped to determine whether they are mutant for the gene of interest, and then may be used experimentally (Gerlai R., 2001).

During the homologous recombination events that allow the vector harboring the mutated gene to be inserted into the ES cell's DNA, DNA that flanks the inserted gene may also be carried forward into the blastula DNA so that these regions become integrated into the knockout mice (Wolfer D. P. *et al.*, 2002). Flanking genes that are linked to the mutated allele can complicate data interpretation. It should be noted that this is only a prominent issue when the genotype of the ES cell (generally 129) differs from the genotype of the female (generally B6) implanted with the blastocyst. In this case, the locus containing the null mutation is flanked by genes from the ES cell genotype, and these genes may then differ from those genes flanking the non-mutated form of the gene in the wild type mice. An example of the impact of flanking genes is that from a study of spatial memory performance which differed between two groups of

chimeric mice, each of which had a 129 inbred strain ES cell genotype and then either a genetic background of 129 or B6 inbred strains. Data from this study suggested that the variety of observed spatial memory phenotypes in the knockout mice was related to flanking genes and not the specific gene investigated (Gerlai R., 1996; Wolfer D. P. *et al.*, 2002).

Conditional approaches demonstrate the effects of a temporarily inactive gene than can be engineered to be tissue-specific, rather than the effect of a gene that is inactive in all sets of tissues and at all times throughout development and later life. This approach offers a solution to several of the issues in knockout animals that have been discussed, such as developmental compensation and the knockout occurring globally (Saunders T. L., 2011). Site-specific conditional mutagenesis relies on DNA recombinase Cre and its recognition of 2 LoxP sites to excise the targeted gene by Cre-mediated recombination during the crossing of a floxed strain with a Cre transgenic strain, such that the target gene becomes inactivated in the Cre expression domain (Friedel R. H. *et al.*, 2011). Once the recombination of Cre and the floxed gene have occurred, the effects are irreversible (Orban P. C. *et al.*, 1992; Belteki G. *et al.*, 2005). The tetracycline-inducible system, in contrast, is reversible and the expression of the transgene can be turned ‘on’ or ‘off’ with the presence or absence of a tetracycline-like antibiotic (doxycycline is often used), which generally is presented in a food or water source (Gossen M. & Bujard H., 1992, 1995).

Other novel conditional gene modification approaches include gene-editing techniques such as the CRISPR-cas systems (clustered, regular interspaced, short palindromic repeats (CRISPR-CRISPR associated protein) and optogenetic tools. The CRISPR-cas system uses targeted genome editing technology by integrating many short conserved repeat DNA sequences into the host genome, which then cleave a specific target region of DNA by homologous recombination (Gasiunas G. *et al.*, 2012; Gasiunas G. & Siksnys V., 2013). Optogenetic tools rely on light-sensitive proteins that can be directly activated or inactivated based on the presence of exogenously applied light. Channelrhodopsin-2, a commonly used

photo-sensitive protein, is sensitive to the frequency of blue light and when applied the resulting behavior and synaptic outputs can be assessed (Madisen L. *et al.*, 2012; Ung K. & Arenkiel B. R., 2012).

**Selectively Bred Lines**

Selective breeding is a useful strategy to create lines of animals that possess genetic differences that influence a trait of interest. If a trait has a genetic component, selective breeding results in an alteration in allele frequencies that are relevant to the level of the phenotype (Falconer Douglas S. & Mackay Trudy F. C., 1996). In mice, a variety of morphological, physiological, and behavioral traits have been examined using uni- or bi-directional selective breeding.

The founding population in a selective breeding project must be comprised of genetically heterogeneous individuals. In some cases, this population has been created by crossing two inbred mouse strains to form an F2 population, but multiple mouse strain intercrosses have also sometimes been utilized to increase genetic diversity. The B6 and D2 inbred strains have been used to create the founding populations for several addiction-related projects, in part, because these strains have been found to be widely divergent for a number of such traits (Phillips T. J. *et al.*, 1998; Fehr C. *et al.*, 2005; Palmer A. A. *et al.*, 2005; Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2012a). When the progenitor strains are known to differ for the selection trait, this increases confidence that a strong response to selection will be realized.

Breeding schemes used to create selected lines include individual or mass, between-family, and within-family selection approaches. In the individual selection approach, all subjects are phenotyped on the trait of interest and are rank ordered and the highest and lowest responding individuals are selected as breeders to create the subsequent generations (Falconer Douglas S. & Mackay Trudy F. C., 1996). In family selection, all individuals from a family are tested, mean values for each family are determined and ranked, and then entire families are kept as breeders based on either high or low family mean. This approach leads to high levels on

inbreeding but is ideal for traits with low heritability (Crabbe J., 1999). In within-family selection, the highest performing male and female members of each high line family and the lowest performing member of each low line family are selected as breeders for subsequent generations. The lowest levels of inbreeding occur during within-family selection because all families contribute equally to each subsequent generation.

The duration of selective breeding is based on the intensity of the response to selection and will be discussed below. Short-term breeding projects utilize mass selection strategies for a small number of selection generations (Belknap J. K. *et al.*, 1997). Because inbreeding levels are higher with mass selection, limiting the number of selection generations also limits the accumulated inbreeding (Belknap J. K. *et al.*, 1997). With mass selection, animals with the most extreme scores on the examined phenotypes are all used as breeders, and thus there is a more rapid change in gene frequency for selection trait relevant alleles, and bidirectional selection response may be maximized before inbreeding is high. Selected lines are suitable for identifying genetically correlated traits, which are other phenotypes that diverge with the selected trait (Henderson N. D., 1989a; Falconer Douglas S. & Mackay Trudy F. C., 1996). When this occurs, some common genes are said to influence both the selected and correlated traits. In contrast to short-term selection projects, long-term selection projects generally exert selection pressure for more than 20 generations, and may utilize within- or between-family selection approaches, or individual selection (Crabbe J., 1999).

In a simple conceptualization of bidirectional selective breeding using a cross of two-inbred strain as the founding population, an average frequency of 0.5 for dimorphic alleles are expected in the heterogeneous founding population. These allele frequencies will hypothetically diverge until they are homozygously fixed in opposite direction in the high and low responding selected lines and additive genetic variance would decrease until the selection limit is reached (Phillips T. J. *et al.*, 2002). The realized response (R) to the applied selection pressure can be measured by taking the difference between the average trait score in the selection generation

and the average trait score from the founding population (Falconer Douglas S. & Mackay Trudy F. C. , 1996; Belknap J. K. *et al.*, 1997; Crabbe J., 1999). The selection differential (S), describes the difference between the average trait score of the animals selected as parents to produce the next selection generation and that the average trait score of the population that the parents were selected from. Heritability of the selection trait is estimated by performing a linear regression of R onto S, such that the slope represents the additive genetic variance that contributes to individual differences. For example, heritability estimated across selection generations $S_0$-$S_{35}$, yielded estimates of $h^2$=0.06-0.08 in the FAST lines mentioned above, and $h^2$=*0.02-0.10* in the SLOW lines *(Phillips T. J. et al., 1991; Shen E. H. et al., 1995)*. These data suggest that 6-8% of the phenotypic variance in ethanol-induced locomotor stimulation in this genetic population could be attributed to additive genetic variance. Additive genetic variance, $V_A$, refers to the average effects of a parent's genes that determine the mean genotypic value of its progeny (Falconer Douglas S. & Mackay Trudy F. C., 1996). However, heritability estimates for these selected lines across a shorter span of time were higher, with estimates of total realized heritability of $h^2$=0.25 after the first generation of selection, suggesting that considerable genetic divergence occurred early in selection (Phillips T. J. *et al.*, 1991). These results are consistent with a waning response to selection over time, which would be expected, as additive genetic variance was exhausted. Short-term selection projects often have large and rapid responses to selection and the selection limit is reached with fewer generations compared to long-term selection strategies. Mice selectively bred for either high or low ethanol locomotor sensitization in a short-term selection project had heritability estimates of $h^2$=0.22 after 4 selection generations, indicating that 22% of the difference in ethanol locomotor sensitization between the selected lines could be attributed to additive genetic variance (Linsenbardt D. N. & Boehm S. L., 2nd, 2013). Additive genetic variance will be largest in the initial heterogeneous founding population and will decrease as trait-relevant genes become homozygously fixed.

Furthermore, the response to selection will occur more rapidly with major effect genes compared to smaller effect genes (Falconer Douglas S. & Mackay Trudy F. C., 1996).

If a different set or a subset of genes influences the selection trait in one direction, but not the other, then frequencies at that locus would change in the “affected” line, but not in the oppositely-selected line. Thus, homozygous fixation for different allele forms at all relevant loci may not necessarily occur. However, in the case of different genetic contributions to the traits in opposite directions, gene frequencies should remain at about 0.5 in one line for those genes not contributing to their selection trait, but be higher for a particular allele in the other line. An example of a selection experiment where trait relevant gene fixation appears to have occurred is in lines of mice bred for high (FAST) ethanol-induced locomotor activation scores in one direction and low (SLOW) scores, including locomotor depression, in the other. When these lines were reverse selected, there was significant movement toward the population mean. During selective breeding experiments, reverse selection places selection pressure in the inverse direction or the trait. In the FAST and SLOW lines, animals with low ethanol-induced locomotor activity in the FAST line and animals with high ethanol-induced locomotor activity in the SLOW line were selected as breeders. If additive genetic variance had been exhausted (i.e., the lines had been homozygously fixed for different alleles at the same loci), then reverse selection should not have been successful (Phillips T. J. *et al.*, 2002). If selection is relaxed and the responses of each selected line on the phenotype begin to regress toward the mean, then it can be assumed that additive genetic variance had not been exhausted in the breeding population and that all trait relevant genes had not been homozygously fixed. However, the absence of a response to selection does not necessarily indicate that all genetic variability at relevant loci has been exhausted (Cunningham D. L. & Siegel P. B., 1978), nor does the lack of regression toward the mean.

Allelic dominance can also affect the genetic outcome of selection, if genetic variance is still present but the population fails to respond to selection pressure. Again, homozygous

fixation may not occur when mice that are homozygous for one allele and mice that are heterozygous at the same locus have similar trait levels (Falconer Douglas S. & Mackay Trudy F. C., 1996). Allelic dominance may be a source of successful reverse selection in selected lines when it was thought that the selection limit had been reached, such that the phenotype for each selected line begins to regress toward the mean. Finally, it should be noted that a selection response may occur in only one direction, and the initial level of response of the founding population should be considered when embarking on a bidirectional selection program, as a floor or ceiling effect could be seen.

Genetic drift, or changes in frequency of alleles in a population, may mask the selection response or result in the loss of selection trait-relevant alleles from the population (Crabbe J., 1999; Palmer A. A. & Phillips T. J., 2002). The act of selectively breeding for trait-relevant genes is inbreeding, but attempts are made to minimize inbreeding for genes not relevant to the selection trait (Crabbe J. C. *et al.*, 1990). This is done by maintaining multiple families and avoiding the mating of close relatives. However, some inbreeding will occur over time. For example, in a short-term selective breeding project for sensitivity to ethanol sensitization, the rate of inbreeding was approximately 1% in each generation for a total of 4% across the 4 selection generations, suggesting that very few genes were homozygously fixed that were unrelated to locomotor sensitization (Linsenbardt D. N. & Boehm S. L., 2nd, 2013). The estimated per generation inbreeding rate for a long-term selection project for high blood ethanol concentration in a drinking in the dark procedure was approximately 1.6% over 11 selection generations for a total of 12.8% (Crabbe J. C. *et al.*, 2009).

Other genetic pitfalls of selective breeding include inbreeding depression, which increases infertility of breeders, unequal selection pressure, founder effects, and bottleneck effects, which can decrease the allelic pool. When an allele frequency is low in a population, there are fewer options for random sampling of that allele and the effect of genetic drift is large. Reduced genetic variation in a breeding population may also result from founder effects, when a

few individuals create a new breeding population and reduce the genetic variation present in the breeding pool. Additionally, bottleneck effects occur when the size of the overall population decreases, reducing genetic variation. This can occur as the result of reduced fertility, adverse environmental effects, or genetic factors that impact survival. Further, indirect selection for a hidden covariate and potential environmental changes can influence the selection response observed in a breeding project (Falconer Douglas S. & Mackay Trudy F. C., 1996).

Though potential pitfalls are discussed above, multiple steps can be taken to reduce the probability of these confounds from occurring. Inbreeding and genetic drift can be reduced by limiting the number of selection generations in short-term selection projects, maintaining relatively large population sizes and avoiding mating of close relatives (Crabbe J., 1999; Palmer A. A. & Phillips T. J., 2002). The inclusion of certain controls during the creation of selected lines also improves their statistical and behavioral interpretation. Potential controls include producing replicated lines and randomly bred control lines not under selection pressure. Additionally, assessment of trait correlations in sets of replicated lines strengthens conclusions about whether the selection and other traits are truly genetically correlated. Replicate lines may be produced either consecutively or simultaneously; however consecutive production of the lines removes the potential influence of environmental factors that might have influenced the selection response (Falconer Douglas S. & Mackay Trudy F. C., 1996), and allows for extension of findings obtained in the previous set of lines.

**Gene Expression**

Gene expression profiles generated using techniques such as microarray or quantitative polymerase chain reaction (qPCR), allow the expression levels of genes to be assessed genome wide, for related sets of genes, or based on the relative expression of a gene in a particular tissue. Microarray techniques often use a genome-wide approach and measure gene expression profiles for thousands of genes or polymorphic markers, in contrast to qPCR chips, which assess multiple genes of interest using a specialized chip that is spotted with nucleotide

sequences that correspond to specific genes. The chip is scanned for the amount of chemiluminescence based on the amount of cDNA bound to each probe on the microarray chip, which gives an indication of relative gene expression levels. Genes within a QTL region that are differentially expressed (DE) between the study populations are more likely to influence the mapped phenotype because the differential expression of a gene may be due to a polymorphism in or near the gene which could be responsible for the observed variation in a behavioral trait (Belknap J. K. *et al.*, 2013). One weakness of qPCR chips is that not all single nucleotide polymorphisms are related to specific genes on the chip.

qPCR measures the expression for a specific gene(s) of interest and does not perform a genome-wide evaluation; it can be quantified for either the absolute or relative expression levels of a gene. Absolute gene expression will be provided in terms of the number of cycles that occur before the fluorescence in a sample cross the baseline fluresence compared to a set of known copy number gene standards. Relative qPCR provides gene expression levels in terms of fold change between a target gene and a background gene, stably expressed between groups. This technique is often the gold standard used to evaluate candidate genes once a QTL region has been mapped more finely and the number of genes has been reduced. In addition, it may be used to confirm differential expression of genes from microarray analyses. For example, a microarray procedure was used to identify DE genes in NAc tissue from drug-naïve HMACT and LMACT mice and found 15 transcripts from the 12,488 probesets evaluated, which differed between the HMACT and LMACT lines. qPCR was used to confirm that 80% of these transcripts significantly differed between the LMACT and HMACT selectively bred mouse lines (Palmer A. A. *et al.*, 2005).

In qPCR methods, weaknesses may include basal differences between the groups of interest in the expression levels of a reference gene that acts as an endogenous control. The control gene is assumed to not be effected by the experimental conditions, but if expression of the housekeeping gene selected is unstable then there could be inconsistencies with

interpreting the expression of the target gene, resulting in false positive or negative data. For example, often the gene, *Gapdh*, is used as a housekeeping gene, but occasionally the gene manipulations performed may also alter the basal levels of the housekeeping gene because of a linked gene effect. In this case, the expression of the target gene may not be accurately detected (Mane V. P. *et al.*, 2008). Other weaknesses observed during qPCR approaches include a limited representation of the total number of gene transcripts and that not all expressed transcripts of a gene may be included. A newer state of the art procedure, RNAseq, uses high throughput deep sequencing techniques that run parallel sequencing to produce large numbers of short sequences which can then be realigned to a reference genome. This approach allows transcribed regions of genes to be sequenced while simultaneously collecting gene expression data, therefore having the ability to provide both sequence and expression data (Wang Z. *et al.*, 2009).

**Genetic Risk for Human Methamphetamine Use**

Not all initial users of MA continue to use MA regularly or develop MA-use disorders. This observation raises the question of whether specific genetic risk factors exist that might predispose certain individuals to develop a MA use disorder. This question is based on the concept of heritability, in which variation in the trait is based on familial genotypic variation (Visscher P. M. *et al.*, 2008). GWAS and linkage studies have suggested roughly 40 candidate genes that may be involved in MA use disorders. Of these candidate genes, 17 had significant genotypic, haplotypic, or allelic associations with MA-dependence, -abuse, or -psychosis (see Bousman *et al.*, 2009 for a full review of candidate genes). Among these gene candidates, 72% have roles in neurotransmitter signaling, metabolism, or release and 13% in MA metabolism or detoxification (Bousman C. A. *et al.*, 2009). Several of the candidate genes with the largest statistical magnitudes of effect are discussed below, with regard to their relevance to MA or AMPH effects.

A meta-analysis identified two appropriately powered GWAS, as indicated by a power analysis, examining MA abuse and dependence. The GWAS highlighted potential involvement of the β-arrestin 2 gene (*ARRB2*) and the prodynorphin gene (*PYDN*). *ARRB2* encodes a scaffolding protein that regulates GPCRs and mediates their desensitization. It appears to have a role in DA signaling, specifically in the AKT-glycogen synthase-3 protein cascade for DA D2 receptors (Beaulieu J. M. *et al.*, 2004). Because of its DAergic regulation and possible involvement in the actions of typical antipsychotic drugs, which function to reduce DA levels, *ARRB2* has been investigated as a risk factor for schizophrenia. However, a genetic case-control study of *ARRB2* in the pathophysiology of schizophrenia and MA use disorders in a Japanese population found a gene-gene interaction for *ARRB2* and *AKT* as risk factors for the development of MA use disorders, but not schizophrenia (Ikeda M. *et al.*, 2006; Ikeda M. *et al.*, 2007).

The *DAT* gene has been implicated by GWAS as a potential candidate to influence the subjective effects of MA. Subjects were genotyped for the DAT 3' untranslated region (UTR) and categorized based on having either 9 or 10 tandem repeats (Lott D. C. *et al.*, 2005). Subjects with 9 tandem repeats reported reduced subjective effects of AMPH, compared to subjects with 10 tandem repeats. This same polymorphism has been associated with reduced responsiveness of children with ADHD to methylphenidate, which has similar pharmacological activity to MA (Stein M. A. *et al.*, 2005; Joober R. *et al.*, 2007). In addition, 9 or fewer repeats in the 3'UTR have been associated with MA-induced psychosis following discontinuation of MA use (Ujike H. *et al.*, 2003). Other genes have also been associated with MA-induced psychosis. In particular, dysbindin-1 (*DTNBP1*) was significantly associated with vulnerability to MA-induced psychosis following chronic MA use (Kishimoto M. *et al.*, 2008; Bousman C. A. *et al.*, 2009; Sim M. S. *et al.*, 2014). However when others examined the *DTNBP1* rs3213207 polymorphism, no associations of the polymorphism with MA-induced dependence, psychosis,

or mania were observed, though a significant association with MA-induced panic attacks was identified (Sim M. S. *et al.*, 2014).

*OPRM1* is an obvious target of investigation for opioid dependence, but has also been examined for its potential influence for other drugs of abuse, including, nicotine, ethanol, and psychostimulants**.** Nearly 100 polymorphisms in the human *OPRM1* gene have been investigated for their association with substance abuse (Kranzler H. R. *et al.*, 1998; Schinka J. A. *et al.*, 2002; Crowley J. J. *et al.*, 2003). The identified A118G polymorphsim, the rs1799971(G) allele of exon 1 of *OPRM1* causes the normally encoded asparagine at residue 40 to be replaced by aspartic acid (Asn40Asp) and potentially causes the loss of an N-glycosylation site. This functional missense mutation occurs in 10-32% of the general population, though frequency is dependent on ethnicity (LaForge K. S. *et al.*, 2000). In postmortem brain samples of unknown ethnicity from the Cooperative Human Tissue Bank, the A118 allele was found to have 1.5-2 fold greater expression compared to the G118 allele and when the coding regions for the A118 and 118G alleles were examined in transfected CHO cells, the G118 allele proved to be a functional variant, with deleterious effects on mRNA and protein yield (Zhang Y. *et al.*, 2005). Specifically, the A118G polymorphism causes β-endorphin to bind to the MOP-r 3 times as strongly. This gene variant has also been linked to MA-induced psychosis and dependence (Ide S. *et al.*, 2006; Deb I. *et al.*, 2010) and was associated with higher subjective ratings of a 10 mg dose of d-AMPH (Dlugos A. M. *et al.*, 2011). The G allele has also been associated with better alcohol treatment response using the MOP-r antagonist, naltrexone (Anton R. F. *et al.*, 2008). In all, these data support a role for MOP-r-mediated systems in substance abuse, including MA abuse. Described in the human clinical literature, *OPRM1* has been associated with MA-induced psychosis and dependence (Ide S. *et al.*, 2006).

**Pharmacological Treatment of Methamphetamine Abuse**

Currently, there are no established United States Food and Drug Administration (FDA)-approved pharmacological treatments for MA dependence or prevention of relapse to MA use.

However, a large number of compounds have been investigated in the preclinical and clinical arenas.

**Dopamine Transporter and Receptor Targeting Drugs**

Modafinil, which is a non-AMPH stimulant, is a currently approved drug for the treatment of narcolepsy (Ballon J. S. & Feifel D., 2006). As measured electrophysiologically, modafinil exerts its pharmacological effects by inhibiting DAT, which potentiates DAergic transmission in the striatum (Federici M. *et al.*, 2013). Preclinical data show that pretreatments of modafinil (2 x 90 or 180 mg/kg; injected 60 min before the 1$^{st}$ and 2$^{nd}$ MA injection) before a binge of MA (3 x 7 mg/kg IP; 3h apart) prevented decreases in the DA metabolite, DOPAC, in the striatum (Raineri M. *et al.*, 2011), and prevented MA-primed reinstatement in rats trained to IV self-administer MA (Reichel C. M. & See R. E., 2010). Several clinical trials suggest that modafinil might effectively reduce cocaine intake and prevent relapse (Dackis C. A. *et al.*, 2003; Anderson A. L. *et al.*, 2009; Dackis C. A. *et al.*, 2012). However, with regard to MA, randomized clinical trials have provided more mixed results, demonstrating no differences between modafinil and placebo treated subjects on measures of use, craving, and sustained abstinence (Heinzerling K. G. *et al.*, 2010; Perez-Mana C. *et al.*, 2013). Further, because of their abuse liability, there is some hesitation about using psychostimulants like modafinil as treatments.

Bupropion, though traditionally prescribed for smoking cessation and as a selective serotonin reuptake inhibitor (SSRI), has also shown some efficacy in maintaining abstinence from psychostimulant use. In addition to blocking SERT, it blocks both DAT and NET and results in more DA in the synaptic cleft, which could reduce some of the negative symptoms experienced during withdrawal that may lead to relapse (Stahl S. M. *et al.*, 2004). A PET scan examined selective DAT-binding of radioligand, 11C-ßCIT-FE, in a cohort of men before initiating and during an 11 d bupropion-dosing schedule. These data demonstrated that bupropion bound to striatal DAT following administration of doses in a therapeutic range. Burpropion inhibited the uptake of the DAT radioligand, and had approximately 26% DAT

occupancy for up to 24h following the final dose of bupropion (Learned-Coughlin S. M. *et al.*, 2003). Additionally, in individuals with acute or modest use of MA in the weeks leading up to an abstinent period, bupropion outperformed placebo, based on the number of MA positive urine samples (Elkashef A. M. *et al.*, 2008; Shoptaw S. *et al.*, 2008; Brensilver M. *et al.*, 2012). Because bupropion binds to DAT, albeit with a different time course and extent than that of drugs of abuse (eg. cocaine binds DAT for short amount of time and has been shown to occupy 65-75% of transporters), it is thought that the efficacy of this drug to reduce MA intake is related to DA actions (Logan J. *et al.*, 1997; Wilcox K. M. *et al.*, 2002).

In addition to pharmacological treatments that are DAT reuptake inhibitors, several DA D1 and D2 receptor antagonist drugs have also been investigated. Aripiprazole, is a newer second-generation antipsychotic atypical drug with a unique pharmacological profile, in that it functions as a partial dopamine agonist and is a partial 5-$HT_{1A}$ receptor agonist and partial 5-$HT_{2A}$ antagonist, and is traditionally prescribed to treat schizophrenia, bipolar disorder, and major depressive disorder (Wilcox K. M. *et al.*, 2002; Mamo D. *et al.*, 2007). Risperidone, is also a second-generation atypical antipsychotic used to treat schizophrenia and mixed manic states of bipolar disorder, but is a full antagonist at D1-like and D2-like DA receptors and also possesses some 5-HT activity. Because of the similarities between schizophrenia and MA-induced psychosis, aripiprazole and risperidone have been hypothesized as effective treatments to assist with patients diagnosed with MA-induced psychotic disorder. A double blind randomized trial of aripiprazole or risperidone for the treatment of AMPH-induced psychotic disorder found that both were effective in reducing psychotic symptoms, though risperidone was more effective at treating positive psychotic symptoms, and aripiprazole was more effective at treating negative psychotic symptoms (Farnia V. *et al.*, 2014). The efficacy of these treatments to reduce psychostimulant drug administration have also been examined in a small clinical trial, which demonstrated that pretreatments of aripiprazole decreased the reinforcing effects of MA

and reduced self-administration of MA when subjects were offered doses of MA they had previously sampled (Stoops W. W. *et al.*, 2013).

**Serotonin Receptor Targeting Drugs**

SSRI drugs have had limited efficacy to reduce MA intake. Most investigated has been ondansetron, a 5-HT-3 receptor antagonist that is commonly used to treat nausea and has the ability to modulate corticolimbic DA levels. However, pretreatment with ondansetron did not attenuate MA-induced DA outflow in the NAc in freely moving rats (De Deurwaerdere P. *et al.*, 2005). In an 8 week controlled clinical trial, the efficacy of daily ondansetron treatment in MA-dependent men and women and a control group was assessed; however, ondansetron was not superior to the control group in reducing measures of MA use, craving, or clinical severity of MA dependence (Johnson B. A. *et al.*, 2008). When investigated for their efficacy to reduce MA intake or craving in MA dependent subjects, SSRIs fluoxetine (Batki S. L. *et al.*, 2000) and sertraline (Shoptaw S. *et al.*, 2006), were not effective.

**y-aminobutryic Acid Receptor Targeting Drugs**

Compounds that act on inhibitory GABA neurotransmitters have also been evaluated, as GABA interneurons in the NAc, which normally hold DA neurons under tonic inhibition, reduce DA levels in the NAc and VTA. Following acute MA administration, levels of DA are increased in the VTA and NAc and GABA is reduced in the NAc, contributing to the reward experienced. It has been hypothesized that GABA receptor agonist drugs would function to increase inhibitory current and decrease DA levels further, thereby making MA less reinforcing. The effects of baclofen, a selective $GABA_B$ agonist and gabapentin, an anticonvulsant that increases GABA levels by inhibition of GABA-transaminase, were examined in a 16 week, placebo-controlled, double blind clinical trial for MA dependent subjects. Baclofen, but not gabapentin, out-performed placebo, with a higher percentage of drug free urine samples and consecutive abstinent periods (Heinzerling K. G. *et al.*, 2006).

**Opioid Receptor Targeting Drugs**

Naltrexone, a MOP-r antagonist, is currently prescribed to manage opioid dependence and alcohol dependence. It targets primarily the MOP-r subtype, but at higher doses is known to possess some activity at κ and δ opioid receptor subtypes (Wang D. *et al.*, 2007). Naltrexone reduced the subjective euphoric effects of an oral dose of AMPH in healthy participants with no history of drug use (Jayaram-Lindstrom N. *et al.*, 2004). Furthermore, in randomized naltrexone vs placebo clinical trials, subjects who received naltrexone had fewer AMPH positive urine samples, a longer period of continuous AMPH abstinence, and less AMPH craving, compared to placebo treated subjects (Jayaram-Lindstrom N. *et al.*, 2005; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b). These data support MOP-r involvement in mediating some of the neurochemical effects of MA. However, in another study, naltrexone did not significantly impact the reinforcing effect of intranasal MA (Stoops et al., 2015). The combination of naltrexone with the partial MOP-r agonist, buprenorphine, has also been evaluated for treatment of poly-drug dependence in users who also have comorbid psychiatric symptoms (McCann D. J., 2008). Because of its partial agonist activity at the MOP-r, there is some concern for the abuse liability of buprenorphine. Additionally, bupropion, a dopamine and norepinephrine reuptake inhibitor and naltrexone in combination have been investigated for their efficacy to reduce the subjective effects of MA. Alone or in combination, neither drug had efficacy to reduce the subjective effects of intranasal MA (Stoops W. W. *et al.*, 2015). Thus, results are somewhat contradictory and further evaluation of MOP-r agonist and antagonist drug effects are needed to determine their ability to effectively treat MA dependence and prevent MA relapse.

**MA Effects in MOP-r Genetically Modified Mice**

A few studies have examined the effects of MA in mice lacking functional MOP-rs. The acute stimulant response and the development of MA-induced behavioral sensitization were attenuated in MOP-r knockout mice (Shen X. *et al.*, 2010). These results are consistent with those demonstrating that MOP-r antagonists can reduce MA-induced locomotor activation in

mice. (Jones D. N. & Holtzman S. G., 1994; Wang J. Q. *et al.*, 1995; Chiu C. T. *et al.*, 2005; Horner K. A. *et al.*, 2010). *In vivo* microdialysis in wild type and MOP-r knockout mice examining the levels of 5-HT and DA metabolites following MA, found dose-dependent decreases in 5-HT and DA metabolites after MA treatment; however, MOP-r knockout mice had a truncated duration of metabolite decrease (Lan K. C. *et al.*, 2008). These findings support MOP-r mediation of MA effects.

A "humanized" transgenic has been created that possesses a particular *OPRM1* variant , consistent with the *OPRM1* A118G SNP identified in humans and discussed above. Since this same gene variant does not naturally occur in mice, a transgenic harboring the equivalent nucleotide/amino acid substitution (*Oprm1* A112G) was generated and has similar phenotypic characteristics as found in association with the human A118G SNP, including reductions in *OPRM1* mRNA levels and in sensitivity to morphine analgesia (Mague S. D. *et al.*, 2009). In addition, [$^{3}$H]DAMGO-binding was used to demonstrate that the A112G SNP was associated with reduced expression of MOP-r in various brain regions, including components of the reward pathway, such as the NAc core, NAc shell, and VTA (Wang Y. J. *et al.*, 2012; Wang Y. J. *et al.*, 2014). However, MA effects in these transgenic mice have not been examined, to the best of my knowledge.

**Lines Selected for MA-related Phenotypes**

MA-related phenotypes have not often been the target of selective breeding projects, but lines have been created for 3 MA-related traits. Bidirectional selective breeding was used to create high and low MA activation (HMACT and LMACT) lines (Kamens H. M. *et al.*, 2005). In addition to a difference in the magnitude of locomotor activation to MA, the HMACT line was also more stimulated than the LMACT line by cocaine. Both lines showed sensitization to cocaine and to a higher dose of MA (2 mg/kg) that was of similar magnitude, but the HMACT line differed in the magnitude of MA-induced sensitization to a lower dose of MA (1 mg/kg), for which a ceiling effect was less probable. Negative genetic correlations with the selection trait,

MA-induced activation, were found for consumption of MA, consumption of cocaine, and locomotor response to cocaine. Thus, the LMACT line had lower locomotor activation to both MA and cocaine, and also consumed more of both drug solutions, compared to the HMACT line, leading to speculations that extreme sensitivity to MA may play a protective role against psychostimulant drug intake. Selected lines have also been created for higher and lower levels of MA-induced locomotor sensitization (MAHSENS and MALSENS) (Scibelli A. C. *et al.*, 2011). MAHSENS mice had a larger acute locomotor stimulant response to MA and consumed lower amounts of MA, compared to MALSENS line mice. These data also support a negative genetic correlation between drug sensitivity and intake.

Our lab has produced 3 replicate sets of bidirectionally selectively bred mouse lines that consume higher or lower amounts of MA in a free-choice two-bottle drinking procedure. Two-bottle choice MA drinking was chosen as the trait for selective breeding, in part, for reasons of feasibility. Other measures of MA-induced reinforcement (e.g., operant IV self-administration) require more complex procedures, which would be difficult to conduct on the relatively large populations of mice that must be tested to generate selectively bred lines (approximately 120 mice per generation). The MA drinking (MADR) selected lines were created using a short-term mass selection procedure. The MADR lines were replicated sequentially, at 2-year intervals, so that research results obtained in one set of lines could be used to generate new hypotheses for follow-up in the subsequent set of lines. Replicate lines have also been used to confirm genetic correlations. Similar results for the selection response, several other MA-related traits, and QTL mapping results (discussed further below) have been obtained in two or more sets of the MADR lines (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Phillips unpublished data). Important existing findings in these lines at the time my thesis project was being developed include the following: MAHDR mice exhibited more robust operant oral and intracranial MA self-administration, compared to MALDR mice; MAHDR mice exhibited MA-induced CPP, which was not seen in MALDR mice; and MAHDR mice showed little sensitivity to conditioned aversive

effects of MA at doses the same or higher than those that induced robust aversive responses in MALDR mice (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2012a; Shabani S. *et al.*, 2012b); a large effect QTL for MA drinking was mapped to mouse chromosome 10. The mapping result (Belknap et al., 2013) was unpublished at the time. In general, the MAHDR mice are sensitive to the rewarding effects of CPP, while the MALDR line mice are sensitive to the aversive effects of MA, both of which likely impact MA intake. Chromosome 10 may harbor one or more genes that impact these traits.

**Genetic Factors Influencing MA intake in Selectively Bred Mice**

To identify the genome-wide location of genes involved in the difference in MA intake between the high and low MA drinking mice, a QTL analysis was performed (Belknap J. K. *et al.*, 2013). DNA samples from the first and second replicate sets of the MADR selected mouse lines were used, and a major effect QTL was identified on mouse chromosome 10 in both sets of lines. This QTL was supported by a ~ 40 Mb interval and accounts for greater than 50% of the genetic variance in MA intake. Many genes reside in this interval, but, as described below, the presence of polymorphisms between D2 and B6 mice and the results of microarray expression analysis in the MADR lines, supported consideration of *Oprm1* as a candidate for the quantitative trait gene (QTG).

The first analysis of potential gene expression differences between the MAHDR and MALDR lines, examined only 384 genes for DE, which were those included on the Mouse Mood StellARray qPCR array (Wheeler J. M. *et al.*, 2009). This qPCR array was chosen because the genes represented on the chip have been associated with those involved in drug-related responses from multiple studies. Mice were administered either saline or 2 mg/kg MA and NAc tissue was taken 4 h later. Large differences in gene expression after saline and in response to MA existed between the replicate 1 MADR line mice, with only 3 genes that were commonly regulated by MA. In some cases, MAHDR had higher expression of a set of genes under saline conditions, and the MALDR line had greater up-regulation or down-regulation of some of these

genes by MA. The opposite results were detected for some other sets of genes, for which the MALDR mice had greater basal expression levels, and greater up-regulation or down-regulation was detected in the MAHDR line (see Wheeler et al., 2009 for a full summary of these data). Existing expression QTL (eQTL) data for BXD RI strains was available in the GeneNetwork public database (www.genenetwork.org) and used to examine potential genetic loci that could influence the expression of genes that were DE on the qPCR chip between the MADR lines. Seven different eQTL were identified on six different Chr that were related to DE genes between drug-naïve mice of the MADR lines (Wheeler J. M. *et al.*, 2009); these represent locations of MADR risk alleles, but are only relevant to those genes represented on the qPCR array.

Though the qPCR data provided interesting potential targets, the analysis was quite restricted. Thus, a more global genome-wide microarray analysis was performed, using tissue from 3 brain regions (mPFC, NAc, and Vmb) and an Affymetrix 430 2.0 mouse gene chip, which includes approximately 45,000 different gene transcripts, representing 19,000 different distinct genes (Belknap *et al*., 2013). For the mPFC, NAc, and Vmb brain regions; 787, 195, and 399 gene probe sets were DE between the MAHDR and MALDR mice. The DE probe sets were distributed across the genome; however, there was an overrepresentation of DE genes in the Chr 10 region, which corresponded to the detected Chr 10 QTL for MA consumption in the lines. One gene in the Chr 10 QTL interval that immediately garnered our attention was *Oprm1* (the many reasons for considering that it might be influential were discussed in previous sections). It should be noted that the above-discussed qPCR chip did not include *Oprm1*; however, in the genome-wide analysis, 1.5-fold greater expression of *Oprm1* was found in the mPFC of MALDR mice, compared to MAHDR mice. The MADR lines did not differ in *Oprm1* expression in the Vmb or NAc. In addition to these data, the Sanger Database indicated that an eQTL exists on Chr 10 between B6 and D2 mice. This Chr 10 eQTL is in the region of the Chr 10 QTL for MA intake; however, it was identified using expression in hippocampal tissue, which has not been analyzed in the MADR lines of mice.

**Experimental Goals and Hypotheses**

Based on the existing genetic findings, the current project sought to identify and characterize opioid mechanisms that may influence oral MA intake in the MADR lines. *Oprm1* was hypothesized to be a QTG that influences the difference in oral MA intake in this genetic animal model. I thus designed experiments to examine opioid-related behavioral, genetic, and pharmacological effects. First, sensitivity to analgesic effects of opioids was measured using thermal and chemical assays. It has been well-established that MOP-rs mediate antinociception and phasic and tonic based assays have been shown to involve differing descending pain mechanisms (Tyers M. B., 1980). It has been suggested that heat-based assays are most sensitive to MOP-r agonist drugs; while non-heat- or chemical-based pain assays activate both MOP-r and $\varkappa$ receptor opioid subtypes and involve changes in mesolimbic DAergic activity (Tyers M. B., 1980; Gear R. W. *et al.*, 1999). It was also advantageous that existing data for these measures, among others, were available in the progenitor B6 and D2 inbred mouse strains, which are also known to differ in brain opioid concentrations, opioid sensitivity, and opioid receptor binding (Belknap J. K. *et al.*, 1989; Belknap J. K. *et al.*, 1990; Belknap J. K. *et al.*, 1995; Belknap J. K. *et al.*, 1998). With regard to sensitivity to morphine-induced analgesia, the D2 strain had greater sensitivity, compared to the B6 strain.

Next, MOP-r drug sensitivity was assessed using a simple locomotor activity procedure. B6 and D2 mice differ in acute MOR-induced locomotor activation, with the B6 strain showing greater activation to MOR compared to the D2 inbred strain (Belknap J. K. *et al.*, 1993a; Phillips T. J. *et al.*, 1994b). I also examined MOR avidity in the MADR lines, using two different drinking procedures that had been previously used to examine this trait in B6 and D2 mice. B6 mice consumed more MOR, compared to D2 mice, in both procedures (Belknap J. K. *et al.*, 1993a; Berrettini W. H. *et al.*, 1994a). This is consistent with IV MOR administration data in which MOR acted as a potent reinforcer for brain stimulation reward in the lateral hypothalamus in B6, but not D2 mice (Elmer G. I. *et al.*, 2010).

Next, I speculated that if greater opioid system activity is related to reduced MA intake in the MALDR mice, then increasing MOP-r activity in the MAHDR mice by stimulation with a MOP-r agonist drug should decrease their MA consumption. Although it was not anticipated that measurable decreases in MA consumption would be observed in MALDR mice treated with MOR-r agonists, due to their already low level of intake, they were nonetheless included in the study. In addition, the effect on MA intake of the MOP-r antagonist, naltrexone, was examined, with the prediction that it would increase MA intake in MALDR mice. However, because naltrexone reduced MA intake in rhesus macaques and had an impact on the subjective effects of MA in human users (Jayaram-Lindstrom N. *et al.*, 2004; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b), it was possible that reduced MA intake might be seen in MAHDR mice after treatment with this drug. Understanding the influence of MOP-r regulated effects on MA intake might justify further examination of novel pharmacological targets to reduce MA dependence. To better understand the temporal relationship of MA intake and the effects of opioid receptor drugs, a lickometer device that measures drinking pattern characteristics was implemented. First, I characterized MA drinking microstructure in the MADR lines of mice. This experiment provided important information about the appropriate time to administer the short-acting opioid agonist, fentanyl, prior to the MA drinking session. Changes in pattern of MA intake following pre-treatment with fentanyl were then examined.

B6 and D2 mice have been previously shown to differ in MOP-r density in several brain regions, with greater density observed in the hippocampus, amygdala, caudate putamen, and whole cortex of B6 compared to D2 mice (de Waele J. P. & Gianoulakis C., 1997). Also, higher affinity binding of the MOP-r agonist, FK 33-824, was observed in brain membrane preparations of B6, compared to D2 mice. None of these studies specifically examined the mPFC, the region where *Oprm1* expression was greater in MALDR than MAHDR mice. I measured MOP-r density in the MADR lines and sought to determine whether the difference in *Oprm1* expression in the

mPFC is associated with a greater Bmax, or total number of receptors, as measured by [$^3$H]DAMGO binding. I examined this in the mPFC, NAc, and Vmb, the same regions examined in the previous microarray expression study (Belknap J. K. *et al.*, 2013), expecting that the MALDR line would have greater MOP-r density, compared to the MAHDR line and that no line differences would be observed in the NAc and Vmb regions, corresponding with the expression results. The progenitor B6 and D2 inbred strains were also evaluated for receptor density, because opioid binding levels in the mPFC had not previously been examined. It was of interest to determine if the MOP-r binding data in the MADR lines and their progenitor inbred strains would correspond with each other in the 3 examined brain regions.

The final goal of my thesis project was to confirm the MA drinking QTL on proximal Chr 10, and potentially reduce the ~10-40 Mb segment supported by a 2-LOD support interval using two congenic strains of mice. This was the final, rather than earlier goal, because the congenic strains did not become available until later in the evolution of this project. The congenic strains that were compared to the D2 background strain for MA intake each had a small segment of the B6 genome from Chr 10 introgressed onto the D2 background genome. One of the two congenic strains contained a B6 segment from 0-7.72 Mb, whereas the second contained a larger segment from 0-20.4 Mb. These congenic strains were originally created for the purpose of finer mapping of the MOR consumption QTL also mapped to proximal mouse Chr 10 (Berrettini W. H. *et al.*, 1994a; Berrettini W. H. *et al.*, 1994b). The introgressed segment in both congenic strains possessed the B6 form of the *Oprm1* gene, located at 6.75 Mb. Therefore, if *Oprm1* is a QTG, both congenic strains should show capture of the QTL, by exhibiting lower MA intake compared to the D2 background strain. This would not prove that *Oprm1* is the QTG, but would indicate that the QTG(s) resides in the 0-7.72 Mb segment. Therefore, it was possible that neither or only one of the congenics would show capture of the MA drinking trait. If neither captured the QTL, this would confirm that *Oprm1* is not a QTG, although its regulation could still influence MA intake.

## CHAPTER 2:

# Opioid sensitivity in mice selectively bred to consume or not consume methamphetamine

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*This manuscript has been published in the journal of Addiction Biology (May 19, 2014 3:370-379).*

**Acknowledgements:** ECE developed hypotheses, designed the studies, analyzed the data, and wrote the manuscript. TJP assisted with hypothesis development, study design, reviewed data analysis, and edited the manuscript. This work was supported by the Department of Veterans Affairs, and NIH grants T32 DA07262, P50 DA018165, and R24 AA020245. The authors would like to thank John Harkness and Harue Baba for assistance in the tail flick and methamphetamine two-bottle choice experiments. The authors have no financial conflicts of interest to disclose.

## ABSTRACT

There has been little investigation of genetic factors and associated mechanisms that influence risk for development of methamphetamine (MA) dependence. Selectively bred mouse lines that exhibit high (MAHDR) or low (MALDR) levels of MA intake in a two-bottle choice MA drinking (MADR) procedure provide a genetic tool for this purpose. These lines were used to determine whether opioid sensitivity and MA intake are genetically associated, since opioid mediated pathways influence some effects of MA. Sensitivity to the analgesic effects of the μ-opioid receptor (MOP-r) agonist fentanyl (0.05, 0.1, 0.2, 0.4 mg/kg) was examined using two acute thermal tests (hot plate and tail flick) and one chronic pain test (magnesium sulfate abdominal constriction). Locomotor stimulant responses to fentanyl (0.05, 0.1, 0.2, 0.4 mg/kg) and morphine (10, 20, 30 mg/kg) were also examined. In addition, MADR was measured in the progenitor strains (C57BL/6J (B6), DBA/2J (D2)) of the F2 population from which the selected lines were generated. The MADR lines did not differ in sensitivity to the analgesic effects of fentanyl; however, MALDR mice exhibited greater locomotor activation than MAHDR mice to both fentanyl and morphine. D2 mice consumed more MA than B6 mice. The line differences for MA consumption and morphine activation recapitulated B6 and D2 strain differences for these two traits, but not strain differences previously found for opioid analgesic responses. These results support a negative genetic correlation between MA consumption and sensitivity to the stimulant effects of opioids and suggest the involvement of MOP-r regulated systems in MA intake.

## INTRODUCTION

Not all initial users of methamphetamine (MA) develop patterns of chronic use. It is possible that genetic factors influence predisposition to escalating MA use and addiction. The MA drinking (MADR) mouse lines were developed to address the question of whether genetic factors influence MA consumption, and subsequently to identify mechanisms that influence MA intake. Two replicate sets of high (MAHDR) and low (MALDR) short-term MA drinking selected lines were serially created from the F2 cross of the DBA/2J (D2) and C57BL/6J (B6) inbred strains, with virtually identical results. After only 4 generations of selection, the MAHDR lines consumed ~ 6 mg/kg MA/18 h, whereas the MALDR lines consumed virtually no MA in a two-bottle choice drinking procedure (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). In addition, MAHDR mice were found to be more sensitive to the rewarding and reinforcing effects of MA and less sensitive to the aversive effects of MA, compared to MALDR mice (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Shabani S. *et al.*, 2012a; Shabani S. *et al.*, 2012b). Other traits that differ between the lines are called genetically correlated traits and share genetic influence with the selection trait (Crabbe J. C. *et al.*, 1990).

The effects of MA are mediated through interactions with several monoamine transporters. DA levels in the nucleus accumbens (NAc)/ventral striatum, a component of the ventral reward pathway, influence drug-induced reinforcement (Di Chiara G. *et al.*, 2004; Taber K. H. *et al.*, 2012). DA in the NAc can be increased indirectly through μ-opioid receptors (MOP-r) residing on y-aminobutryic acid (GABA) interneurons in the ventral tegmental area (VTA). MOP-r activation results in hyperpolarization of these interneurons, thereby reducing inhibitory input to VTA DA neurons and enhancing DA transmission in the NAc (Johnson S. W. & North R. A., 1992). In support of opioid involvement in MA reward, human studies have identified an association between a polymorphism in the MOP-r gene, *OPRM1,* and MA-induced psychosis and dependence (Ide S. *et al.*, 2004a; Ferraro T. N. *et al.*, 2005; Ide S. *et al.*, 2005; Doyle G. A. *et al.*, 2006; Doyle G. A. *et al.*, 2007). Furthermore, attenuation of MA-induced behavioral

sensitization and locomotor activation was found in *Oprm1* knockout mice or after pretreatment with MOR-r antagonists (Jones D. N. & Holtzman S. G., 1994; Chiu C. T. *et al.*, 2005; Shen X. *et al.*, 2010). However, the involvement of MOP-r in MA intake has not been examined in animals, and positive results could be relevant to the development of pharmacological treatments to reduce MA consumption.

Because research in both humans and animals has implicated MOP-r mediated systems in MA-related traits, we explored opioid drug sensitivity in the MADR lines. Such differences would suggest that opioid systems were changed in concert with selective breeding for MA consumption, and thus, might play a role in MA intake. We used nociception and locomotor traits as sensitive measures of opioid response to examine opioid drug sensitivity in the MADR lines. More than one measure of sensitivity to analgesic drug effects was used, based on different characteristics of the tests and possible differences in the underlying mechanisms associated with analgesic responses in the tests. The abdominal writhing test provides a model of more chronic pain, compared to the hot plate and tail flick tests, and has been suggested to involve DA pathways (Barton C. *et al.*, 1980; Basbaum A. I. & Fields H. L., 1984; Altier N. & Stewart J., 1999). Because the MADR lines differ in sensitivity to the rewarding effects of MA, we hypothesized that DAergic mechanisms are involved and that the MADR lines would be more likely to differ in opioid sensitivity in the writhing test. We also considered that similar differences between the selected lines and the inbred strains from which they were derived would provide additional evidence of genetic relationships between MA consumption and opioid drug sensitivity. Existing data show that D2 mice have greater sensitivity to the analgesic effects and reduced sensitivity to the activating effects of morphine (Belknap J. K. *et al.*, 1989; Phillips T. J. *et al.*, 1994a; Bergeson S. E. *et al.*, 2001). We examined MA consumption in the B6 and D2 strains, which had not been previously characterized.

## Materials and Methods

### Hot plate test

The hot plate assay examines a coordinated muscle response to a heated stimulus, during which a mouse licks its hind foot to terminate a trial (Mogil J. S. *et al.*, 1996). Each mouse was weighed and placed individually into the cell (8 x 19 x 8 cm) of an acrylic plastic isolation chamber that had holes for ventilation. These chambers provide quick access to each animal, and help to equate body temperature prior to conducting the thermal test by preventing huddling. Mice were housed in these chambers for 10 min and then injected with saline and returned to the chambers for 10 minutes. For hot plate testing, each mouse was placed onto a 10 x 10 cm aluminum hot plate that was maintained at 53□C. A bottomless box with 15 cm high acrylic plastic walls was used to prohibit escape from the hot plate. Latency to first rear paw lick (in seconds) was used as the nociceptive response (Bryant C. D. *et al.*, 2009c). Immediately after the mouse displayed this response, it was removed from the hot plate and returned to its assigned cell in the holding chamber. If no response occurred within a cut-off time of 90 seconds, the mouse was removed from the hot plate to prevent tissue damage and the maximum score of 90 seconds was recorded. Selection of temperature and cut-off latency was based on previous work (Belknap et al., 1995; Bryant et al., 2009b). Two minutes after saline hot plate testing, each mouse was injected IP with fentanyl (0.05, 0.1, 0.2 or 0.4 mg/kg), returned to the holding chamber for 10 minutes, and then tested on the hot plate exactly as described after saline treatment. Doses were selected from previous work (Bryant C. D. *et al.*, 2009a; Romero A. *et al.*, 2010). The measure of drug response was percent of maximum possible effect (% MPE), which was generated using the following formula: (fentanyl latency – saline latency)/(cut off latency – saline latency). This data transformation corrects for differences in saline latency and approximates a more normal distribution (Belknap J. K. *et al.*, 1989; Belknap J. K. *et al.*, 1995). Mice were tested in 4 passes (n = 6-12 per line and sex per pass).

**Tail flick test**

A protracted period of 7 days was allowed post hot plate testing to ensure complete drug elimination and to reduce the possibility of a prior exposure effect. The same mice were then

examined for sensitivity to the analgesic effects of the same dose of fentanyl that they had received before, using the tail flick test. This assay measures a simple nociceptive reflex, involving reflexive movement of the tail to avoid a targeted heat source (Mogil J. S. *et al.*, 1996). Mice were weighed and placed in isolation chambers for 10 min, then injected with saline and returned to the chambers for 10 minutes. Each mouse was then gently restrained, and the distal half of its tail was dipped into a water bath (12 L capacity; 32.7 x 30 x 15 cm; VMR Lab Shop, Radnor, PA) maintained at 49□C. Latency to withdraw the tail from the water (in seconds) was recorded and if the mouse did not withdraw its tail within 15 seconds, the test was terminated to prevent tissue damage. In this case, the mouse was assigned the maximum score of 15 seconds. This test was repeated three times, with an inter-trial interval of 20 seconds, during which time the tail was dried off using a paper towel. Three trials were used based on research showing that three averaged trials improve response accuracy (Mogil J. S. *et al.*, 1995). After completing the three trials, mice were returned to the isolation chambers. Two min after saline tail flick testing, each mouse was injected with 0.05, 0.1, 0.2, or 0.4 mg/kg fentanyl, returned to the isolation chamber for 10 min, and then tested again for tail flick latency. The measure of drug response was % MPE, as described for the hot plate test.

**Magnesium sulfate abdominal constriction test**

Drug- and experiment-naïve mice were used. The abdominal constriction test involves administration of a noxious substance that produces a writhing response (Hendershot L. C. & Forsaith J., 1959). Isolation chambers were used as described above. Mice received injections of either saline or fentanyl (0.05, 0.1, 0.2, 0.4 mg/kg), were returned to holding chambers for 10 minutes, and then given an injection of magnesium sulfate (120 mg/kg), which is known to induce abdominal constrictions or “writhes” (Luo P. *et al.*, 2010), defined as a lengthwise stretch of the torso with a concomitant concave arching of the back. The number of abdominal writhes was counted during a 5-minute observation period. Mice were tested in 3 passes (n = 7-15 per line and sex per pass).

**Locomotor response to fentanyl**

Locomotor activity was measured using eight automated Accuscan activity monitors (Accuscan Instruments, Colombus, OH). Eight pairs of infrared beams and detectors mounted 2 cm above the clear acrylic plastic test chamber (40 x 40 x 30 cm) floor detected locomotion. Photocell beam breaks were automatically recorded and translated by Accuscan software to distance traveled in centimeters. The monitors were enclosed in illuminated (3.3 Watt incandescent bulb) and ventilated environmental control chambers that provided sound attenuation (AccuScan Instruments).

The first cohort of animals tested was that used in the thermal nociception assays, and was tested 7 days after nociception testing. Because of possible effects of prior testing and exposure to fentanyl, this study was repeated in a separate group of mice, which were drug- and experiment-naïve. Only female mice were available for the second study; however, there was no significant effect of sex in the initial study (n=7 per line and dose). On each day of testing, animals were moved to the procedure room for a 45-60 minute acclimation period before testing began. On each day, mice were injected and placed individually into the center of the activity monitors, where distance traveled was measured for 30 minutes, with data collected in 5-minute time bins. On days 1 and 2, mice were treated with saline, and on day 3, they were treated with 0.05, 0.1, 0.2 or 0.4 mg/kg fentanyl. Day 1 allowed habituation to the novel environment and experimenter handling, data collected on day 2 provided baseline activity data, and data collected on day 3 provided a measure of drug response. Day 2 baseline data were subtracted from day 3 drug data for each individual animal to obtain a measure of change in baseline behavior induced by drug treatment, a measure that has been used in several of our previous studies (Phillips T. J. *et al.*, 1992b; Phillips T. J. *et al.*, 1995).

**Locomotor response to morphine**

Drug- and experiment-naïve mice were used to examine sensitivity to the activating effects of morphine (10, 20 and 30 mg/kg). All experimental details were identical to those

described for fentanyl. Doses were chosen from previous work (Phillips T. J. *et al.*, 1992a). Mice were run in 4 passes (n=5-9 per sex and line per pass).

**Two-bottle choice MA drinking**

The identical procedure used for selection of the MADR lines (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011) was used. B6 and D2 mice were isolate housed and given 24-h access for two days to two water-filled 25-ml drinking cylinders, fitted with sipper tubes, to familiarize them with the drinking apparatus. Over the following 8 days, one tube was filled with tap water and one with MA in tap water (20 mg/l for 4 days, then 40 mg/l for 4 days). Mice had 24-h access to water throughout the study, but 18-h access to MA (MA tube removed during hours 3 through 9 of the light cycle). The positions of the tubes were alternated every 2 days to control for side preferences. Body weight was measured every 4 days and used to calculate amount of MA consumed in mg/kg. Volume changes in the tube to the nearest 0.2 ml were used to calculate consumption and preference ratio (ml MA: total ml from both tubes during the same 18-h period) by averaging the second and fourth days for each concentration of MA.

**Morphine clearance**

To examine possible differences in morphine levels and clearance that might contribute to behavioral differences, male MAHDR-2 and MALDR-2 mice were injected IP with 20 mg/kg morphine and blood morphine levels were assessed in independent groups at 15, 30, 60, and 120 minutes after treatment (n=5-7/line/time point). This dose of morphine was chosen as a modest dose, with significant behavioral effects to which the MADR lines responded differently in a study of locomotor activation (see Fig. 2.4 in the associated manuscript). Blood (20 µl) was collected from the retro orbital sinus, using a calibrated glass capillary tube (Kimble Glass Inc.; Vineland, NJ). Each sample was placed into a microcentrifuge tube that contained 80 µl of Neogen (Lexington, KY) EIA buffer (a phosphate buffered saline solution, containing bovine serum and a preservative) and trace quantities of morphine from blood samples were assessed using the Neogen opiate group enzyme-linked immunosorbent assay (ELISA) kit. Samples

were read with a Bio-Rad Benchmark Plus microplate spectrophotometer (Hercules, CA) that was equipped with a 450 nm filter. Morphine concentrations were determined using a calibration curve.

**Fentanyl clearance**

Fentanyl levels and clearance were similarly assessed, using an independent group of male MAHDR-2 and MALDR-2 mice that were injected IP with 0.4 mg/kg fentanyl. Blood fentanyl levels were assessed in independent groups at 15, 30, 60, and 120 minutes after treatment (n=5-7/line/time point). This dose had differential locomotor stimulant effects in the two selected lines of mice. Blood was collected as described for the morphine clearance study and samples were processed using the Neogen fentanyl ELISA kit, and analyzed and quantified as described for the morphine clearance study.

**Data analysis**

Data from the two-bottle choice drinking procedure, abdominal constriction, and locomotor studies were analyzed by factorial Analysis of Variance (ANOVA) with repeated measures when appropriate, using Statistica software (Statsoft Version 9, Tulsa, OK). Possible independent variables were line, sex and drug dose or concentration, and in the case of locomotor activity, both baseline scores and drug response scores were analyzed. Two-way interactions were resolved using simple main effects analysis and the Newman Keuls test was used for post-hoc mean comparisons. Latency to lift or shake the hind paw in the hot plate test or to withdraw the tail in the tail flick test were transformed to % MPE to correct for differences in saline baseline latencies and were analyzed by Kruskal Wallis non-parametric ANOVA, because the dependent variables included cut-off latencies. Independent variables were line, sex and dose, and both latencies after saline and drug response scores were analyzed. Figures were created using Sigmaplot 2002 for Windows Version 8.0 (SPSS, Chicago, IL). All values are

expressed as mean ± standard error of the mean (SEM). The criterion for significance was set at $p<0.05$.

## RESULTS

### Sensitivity to the analgesic effects of fentanyl on thermal nociception in MADR mice

Fig. 2.1 illustrates analgesic responses to fentanyl for the hot plate (Fig. 2.1A) and tail flick (Fig. 2.1B) tests. Data are combined for the two sexes because there were no significant effects of sex. There were no significant line differences in either baseline (saline) nociceptive responses or sensitivity to fentanyl (hot plate saline latencies; mean ± SEM: MAHDR-2: 17.7 ± 0.9 sec; MALDR-2: 17.8 ± 1.0 sec; tail flick saline latencies; mean ± SEM: MAHDR-2: 3.3 ± 0.2 sec; MALDR-2: 2.9 ± 0.1 sec). There was a significant main effect of fentanyl dose for both the hot plate test ($H(3, N=167)=91.6$; $p<0.001$) and tail flick test ($H(3,N=166)=98.4$; $p<0.001$). In addition, a similar percentage of each of the MADR lines was assigned the hot plate cut-off latency of 90 seconds (MAHDR-2: 45.7%; MALDR-2: 45.8%) and the tail flick cut-off latency of 15 seconds (MAHDR-2: 26.5%; MALDR-2: 28.2%). Although the same animals were tested in these two assays, only 51% of the ones that reached the cut-off latency in the hot plate test were the same ones to reach the cut-off latency in the subsequent tail flick test. Although hyperalgesia has been reported with repeated fentanyl administration, this effect was reported to occur with greater exposure and to fully dissipate within a shorter time frame (within 5 days; (Celerier E. *et al.*, 2000) than the 7 days between treatments used here.

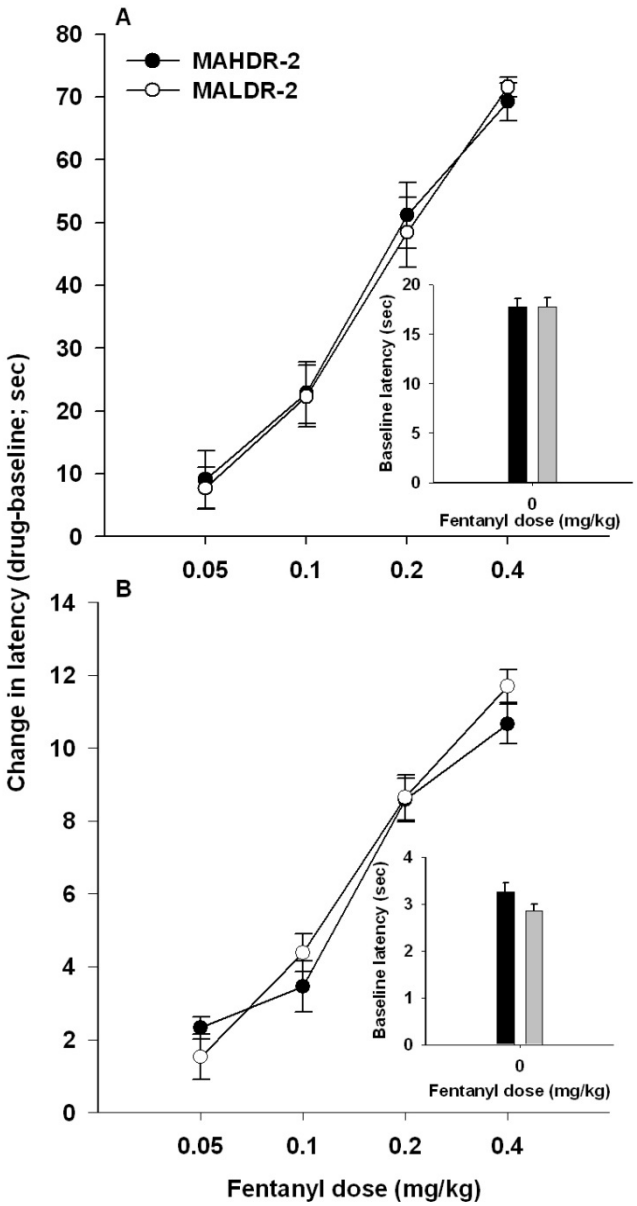


**Figure 2.1. Sensitivity to the analgesic effects of fentanyl in MADR mice using the hot plate and tail flick tests.** Shown are means (± SEM) for the analgesic response to several doses of IP fentanyl for the (A) hot plate and (B) tail flick tests. Means are shown as change in latency between fentanyl latency and saline latency, where 90 and 15 seconds were the cut off latencies for hot plate and tail flick, respectively.

**Sensitivity to fentanyl in the abdominal constriction test in MADR mice**

Fig. 2.2 shows the effects of fentanyl on magnesium sulfate-induced abdominal writhing. Female mice were more sensitive than male mice to the analgesic effects of fentanyl in this test ($F(1,93) = 5.51$; $p<0.05$ for the main effect of sex), but there were no interactions of sex with line or dose; thus, data are shown collapsed on sex. No significant line difference was detected for the number of abdominal writhes. There was a main effect of dose ($F(4,93) = 25.18$; $p<0.01$), indicating that fentanyl dose-dependently reduced the number of magnesium sulfate-induced abdominal writhes.

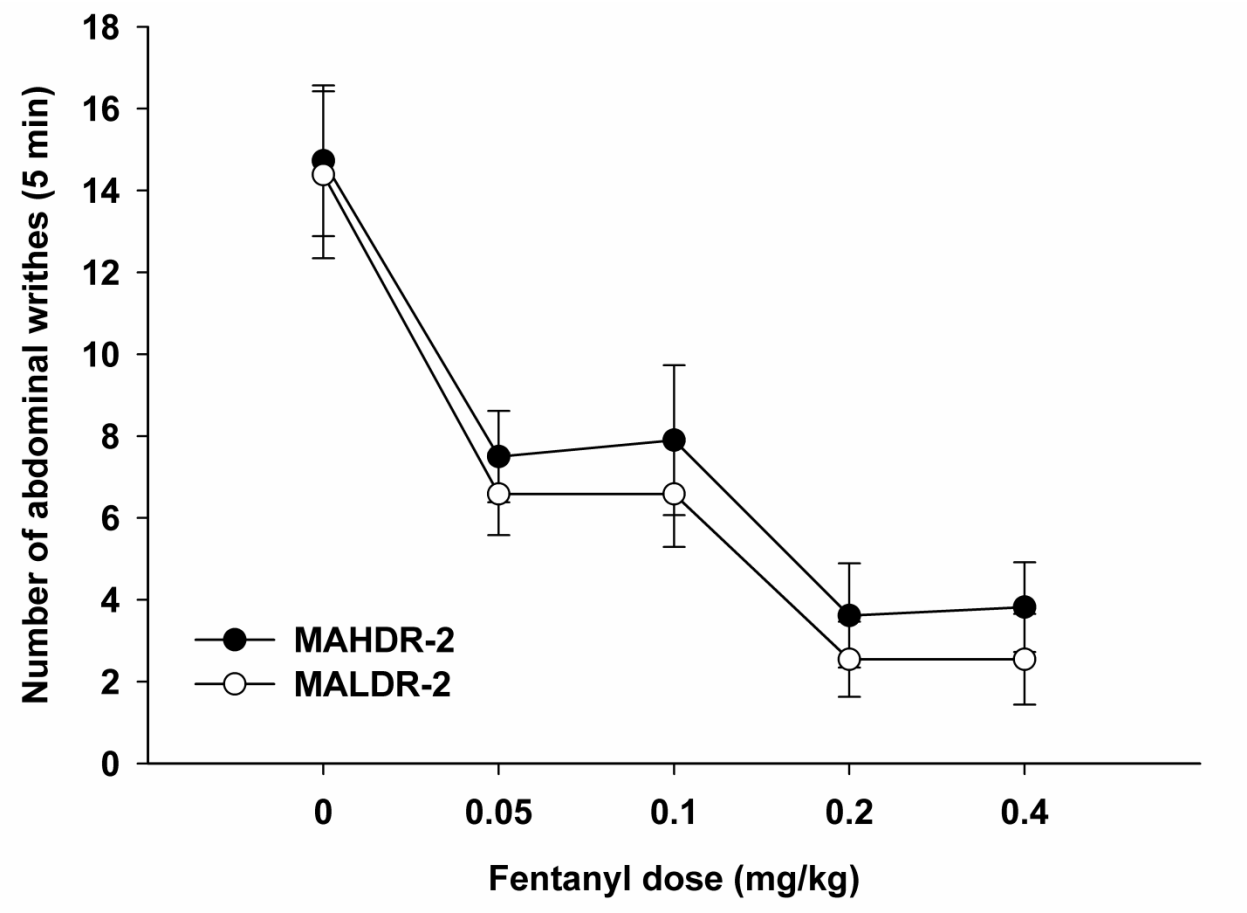


**Figure 2.2. Sensitivity to the analgesic effects of fentanyl in MADR mice using the magnesium sulfate-induced abdominal writhing test.** Shown are mean (± SEM) number of abdominal writhes (constrictions) during a five-minute observation period after IP injection of 120 mg/kg magnesium sulfate. Mice received pretreatment with IP saline or fentanyl, 10 minutes before magnesium sulfate treatment.

**Sensitivity to the locomotor activating effects of fentanyl**

Analysis of baseline locomotor activity data collected on day 2 demonstrated that the low line had greater baseline activity compared to the high line ($F(1,151)= 11.65$; $p<0.05$; mean ± SEM: 5405 ± 204 cm for MALDR-2 and 4271 ± 175 cm for MAHDR-2). The drug response scores (day 3 minus day 2) shown in Fig. 2.3 corrected for this difference. Data for locomotor response to fentanyl were initially analyzed with time as a repeated measure; however, the results with regard to line, sex and dose were comparable to those when data were analyzed for the total 30-minute session. There were no main or interaction effects involving sex, so data are shown for the total session with the sexes combined. Analysis of data from the cohort of mice used in the nociception assays (Fig. 2.3A), demonstrated a greater stimulant response to fentanyl in MALDR-2 than MAHDR-2 mice. There were significant main effects of line ($F(1,51)=8.1$; $p<0.01$) and dose ($F(3,151)=33.4$; $p<0.001$). Analysis of data from mice that were fentanyl- and experiment-naïve (Fig. 2.3B), revealed largely comparable differences, although in this case, the selected lines did not differ significantly in baseline activity level (mean ± SEM: 6144 ± 362 cm for MALDR-2 and 5417 ± 577 cm for MAHDR-2). There were significant main effects of line ($F(1,38)=18.4$; $p<0.001$) and dose ($F(2,38)=30.4$; $p<0.001$), as well as a significant line x dose interaction ($F(2,38)=4.5$; $p<0.05$). Simple effect analysis indicated that MALDR-2 mice were more stimulated than MAHDR-2 mice by both doses of fentanyl.

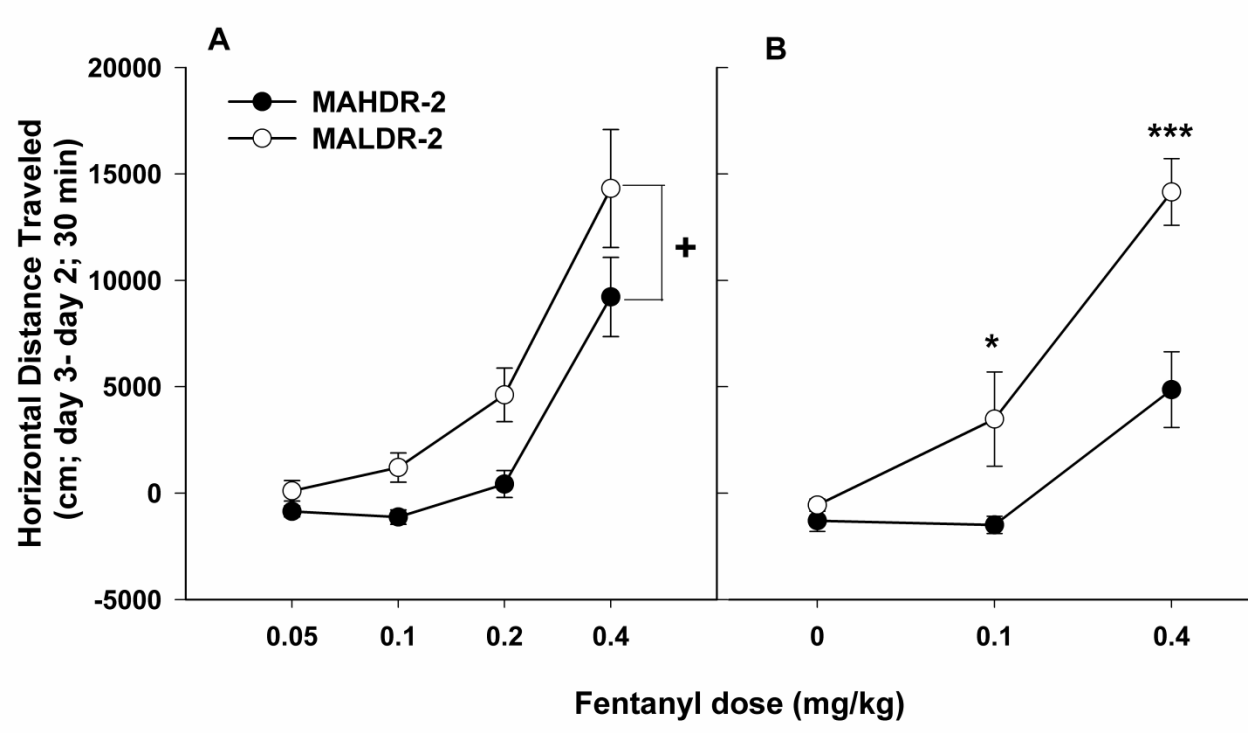


**Figure 2.3. Sensitivity of MADR mice to the locomotor stimulant effects of fentanyl.** Shown are mean (± SEM) difference scores for IP saline and several doses of fentanyl, created by subtracting horizontal distance traveled on saline day 2 from distance traveled on drug day 3 during 30-min locomotor activity sessions. Data shown in (A) are from mice that had been used to obtain the nociception data shown in Fig. 2.1. Data shown in (B) are from experiment- and fentanyl-naïve mice. +p<0.05 for the main effect of line. *p,0.05 and ***p<0.001 for the line difference at the indicated dose.

**Sensitivity to the locomotor activating effects of morphine**

After obtaining significant results for fentanyl, this study was performed to determine whether the selected line results would recapitulate existing results for morphine in the B6 and D2 strains. Analysis of day 2 baseline locomotor activity demonstrated that MALDR-2 mice had greater baseline activity compared to MAHDR-2 mice ($F(1,80)=7.01$; $p<0.05$; mean ± SEM: 5828 ± 273 cm for MALDR-2 and 4823 ± 275 cm for MAHDR-2). The drug response scores shown in Fig. 2.4 corrected for this difference. Data were initially analyzed including time as a repeated measure; however, the results were comparable when data were analyzed for the total 30-minute session. There were no main or interaction effects involving sex, so data are shown for the total session with the sexes combined. MALDR-2 mice were more sensitive than MAHDR-2 mice to the locomotor stimulant effects of morphine. There were significant main effects of line ($F(1,88)= 29.8$; $p<0.001$) and dose ($F(3,88)=6.5$; $p<0.001$) and a significant line x dose interaction ($F(3,88)=3.7$; $p<0.05$). Simple effect analysis indicated that MALDR-2 mice were more stimulated than MAHDR-2 mice by all doses of morphine.

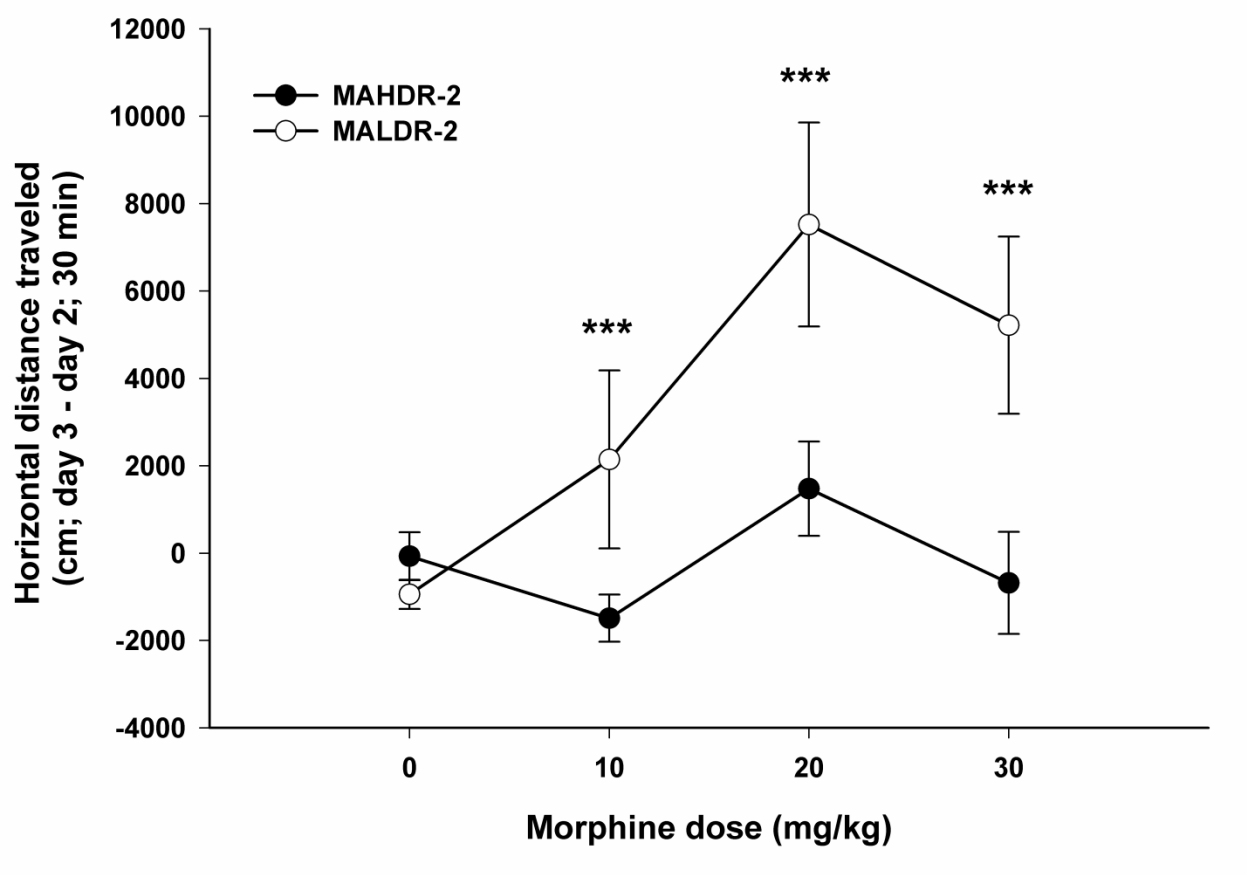


**Figure 2.4. Sensitivity of MADR mice to the locomotor stimulant effects of morphine**. Shown are mean (± SEM) difference scores for IP saline and several doses of morphine, created by subtracting horizontal distance traveled on saline day 2 from distance traveled on drug day 3 during 30-min locomotor activity sessions. ***p< 0.001 for the line difference at the indicated dose.

**MA drinking in B6 and D2 mice using a two-bottle choice test**

As shown in Fig. 2.5A, D2 mice consumed more MA than B6 mice. Repeated measures ANOVA revealed significant effects of strain ($F(1,31)= 84.2$; $p<0.001$) and concentration $F(1,31)=19.4$; $p<0.001$), and a significant strain x concentration interaction ($F(1,31)=18.7$; $p<0.001$). Simple effects analyses indicated significantly greater MA consumption in D2 compared to B6 mice for both concentrations. MA consumption in D2 mice was increased with increasing concentration. A similar strain difference was found for MA preference ratio (Fig. 2.5B). There were significant main effects of strain ($F(1,31)= 81.7$; $p<0.001$) and concentration ($F(1,31)= 15.5$; $p<0.001$), with D2 mice having larger preference ratios compared to B6 mice, and B6 mice showing considerable avoidance of the MA solutions. For total volume (ml/kg) during the 18-h period when both MA and water tubes were offered (Fig. 2.5C), significant main effects of strain ($F(1,31)=7.6$; $p<0.05$), sex ($F(1,31)= 10.8$; $p<.05$) and concentration ($F(1,31)=25.5$; $p<0.001$) were found. D2 mice consumed greater total volume compared to B6 mice (234.2 ± 10.6 ml/kg for D2 and 203.8 ± 8.8 ml/kg for B6) and female mice consumed more total volume compared to male mice (235.5 ± 11.6 ml/kg for female and 199.2 ± 6.4 ml/kg for male). More total volume was consumed when the higher concentration of MA was available compared to when the lower concentration was available (227.7 ± 7.2 ml/kg for 40 mg/l and 205.9 ± 7.1 ml/kg for 20 mg/l).

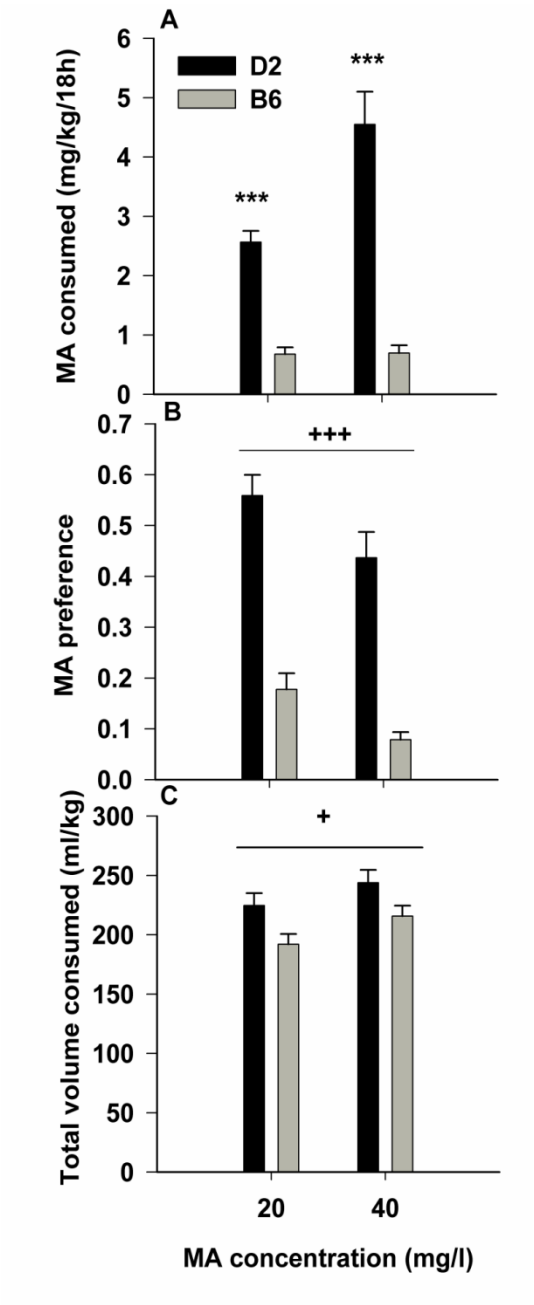


**Figure 2.5. Consumption of MA in D2 and B6 mice in a two-bottle choice drinking procedure.** Data shown are (A) average MA consumed (mg/kg) on the second and fourth days of access to the 20 mg/l and 40 mg/l MA solutions, (B) average drug preference using data from the same days, calculated as the ratio of the volume of drug solution to the total volume of fluid consumed, and (C) average total volume consumed on the same days, corrected for body weight (ml/kg) . All values are expressed as mean ± SEM. ***p<0.001 for the strain difference at

each concentration. +p<0.05 and +++p<0.001 for the main effect of strain. D2: DBA/2J; B6: C57BL/6J

**Morphine clearance**

As shown in Fig. 2.6, the time for morphine to be cleared from the blood was similar between the MADR lines. The MADR lines did not differ in peak morphine blood levels (MAHDR-2: 425.6 ± 103.1 ng/ml; MALDR-2: 356.9 ± 58.8 ng/ml).

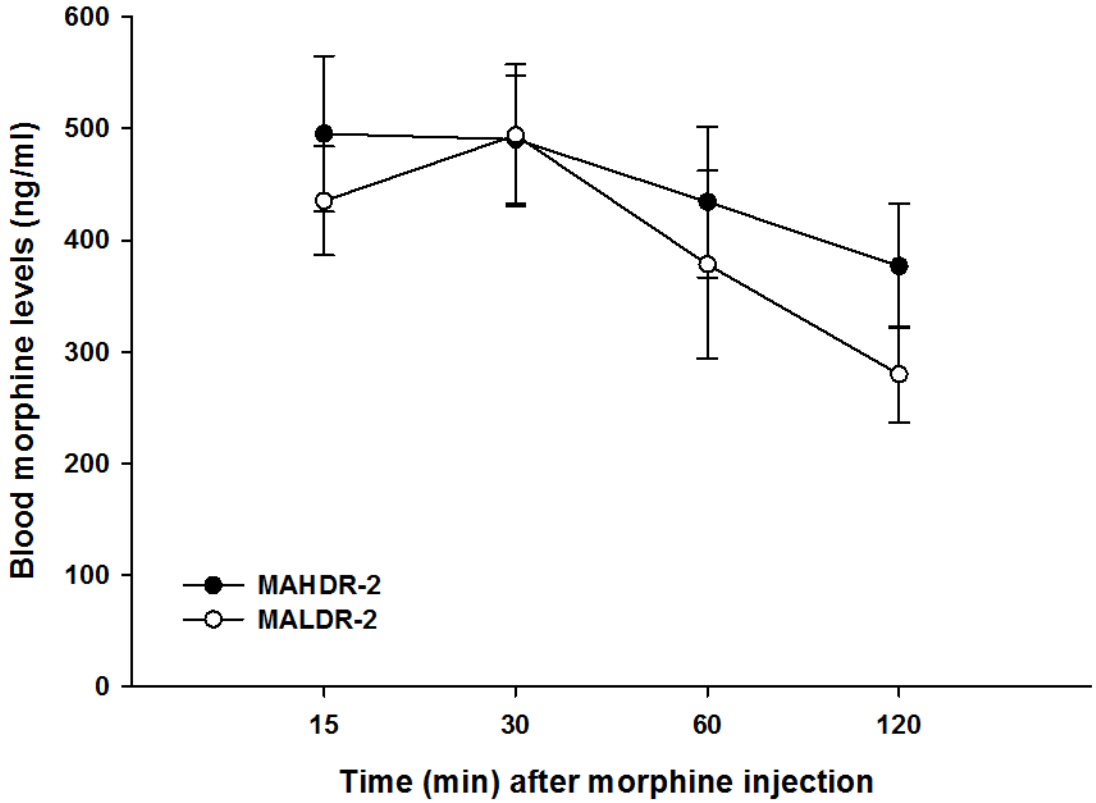


**Figure 2.6.** Morphine clearance between MADR-2 mice. Blood morphine levels in MAHDR-2 and MALDR-2 mice at the time points listed along the x axis, following IP injection of 20 mg/kg morphine. N=5-7/line/time point.

**Fentanyl clearance**

As shown in Fig. 2.7, the time for fentanyl to be cleared from the blood was similar between the MADR lines. The MADR lines did not differ in peak fentanyl blood levels (MAHDR: 27.0 ± 3.6 ng/ml ; MALDR: 26.1 ± 4.4 ng/ml).

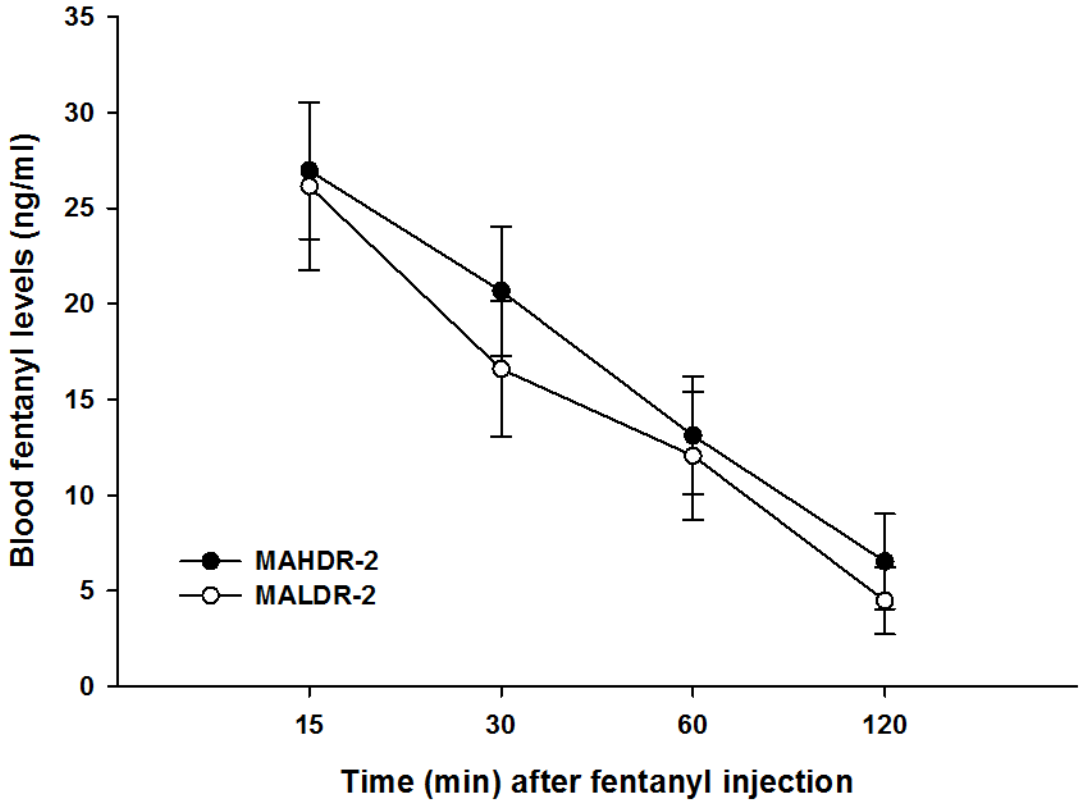


**Figure 2.7.** Blood fentanyl levels in male MAHDR-2 and MALDR-2 mice at the time points listed along the x axis, following IP injection of 0.4 mg/kg fentanyl. N=5-7/line/ time point.

## DISCUSSION

Selective breeding for high and low MA consumption has altered locomotor sensitivity, but not analgesic sensitivity, to MOP-r agonist drugs. MA consumption levels in the B6 and D2 mice indicate that the MAHDR lines are more D2-like for MA intake, while the MALDR lines are more B6-like. The MADR line difference also recapitulated this strain difference for locomotor stimulation by opioid agonist drugs; D2 and MALDR-2 mice were more stimulated than their respective B6 and MAHDR-2 counterparts (data shown here and in Belknap et al., 1989). Overall, we show a negative genetic correlation between sensitivity to the locomotor activating effects of MOP-r agonist drugs and MA consumption, providing some evidence for a change in MOP-r mediated systems as a result of selective breeding for high vs. low MA intake.

Differences in behavioral stimulation between the high and low MADR lines were found for both fentanyl and morphine. We initiated our studies using fentanyl because it has been shown to be more selective for the MOP-r subtype, while morphine acts on both μ- and κ-, but not δ- opioid receptor subtypes. Although morphine is less selective, it has higher affinity for the μ than for the κ receptor (Raynor K. *et al.*, 1994; Kalvass J. C. *et al.*, 2007). Fentanyl and morphine have similar affinity for the MOP-r, however fentanyl is 100 times more potent, compared to morphine, and crosses the blood brain barrier with greater ease (Volpe D. A. *et al.*, 2011). Though these opioid receptor drugs have different potencies and specificity for the opioid receptor subtypes, they both produced locomotor stimulation and similar differences in response were seen between the selected lines. This supports a role for differences in MOP-r-mediated effects between the two lines. Concerns about drug carryover effects influencing the line difference results seen for fentanyl-induced locomotion led to evaluation in experimentally naïve animals. Similar results were observed in both cases, with MALDR-2 line mice showing greater locomotor activation to fentanyl compared to MAHDR-2 mice. Completed studies indicate that the MADR lines do not differ in peak levels or clearance of morphine or fentanyl, suggesting that

differences in sensitivity, rather than pharmacokinetic factors, are responsible for the observed behavioral differences.

Significant baseline locomotor activity differences were found for two of the three locomotor activity studies, with higher levels of baseline activity in MALDR-2 than MAHDR-2 mice. There was a tendency toward a similar difference in the third study. Clearly this difference does not account for the line difference in opioid response because (1) the drug response data were corrected for baseline activity level and (2) one might expect the line with the lower activity level (the MAHDR-2 line) to be able to show greater activation, yet the opposite result was obtained.

No differences were found between the MADR lines in sensitivity to the analgesic effects of fentanyl. Pain has been placed into two categories; phasic and tonic pain. The first refers to pain spanning minutes or less, such as that induced in the hot plate and tail flick tests, which are terminated with a simple reflexive response. Tonic pain, refers to pain of greater duration, such as that associated with the acetic acid and magnesium sulfate abdominal constriction tests (Barton C. *et al.*, 1980). Analgesic drug effects in phasic pain tests have been suggested to act through direct spinal pathways that activate opioid receptors in the periaquaductal grey (PAG) brainstem region, whereas analgesic drug effects in tonic pain tests are thought to involve changes in mesolimbic DA activity in the VTA via opiate actions (Barton C. *et al.*, 1980; Basbaum A. I. & Fields H. L., 1984; Altier N. & Stewart J., 1999). We hypothesized that the tonic pain assay would be more likely to detect a line difference in opioid sensitivity, if MA consumption were being mediated through the indirect action of opioids on DA pathways. However, the current results suggest that sensitivity to the analgesic effects of fentanyl in both pain assays does not share a common genetic mechanism with MA consumption.

Data for MA preference drinking have not been previously published for the D2 and B6 inbred mouse strains. We predicted that each of the selected lines might resemble one of the inbred strains for MA consumption, based on rapid response to selection, suggesting the

possibility of a major gene effect on MA intake (Shabani S. *et al.*, 2011). The D2 strain consumed more MA, making them more similar to the MAHDR lines, while the B6 strain consumed low amounts of MA, making them more similar to the MALDR lines. The D2 mice also consumed more total fluid volume; however, this difference was small compared to the large magnitude difference in MA consumption between the inbred strains. A similar strain-line association was found for sensitivity to opioid-induced locomotor activation. B6 mice have been found to show greater locomotor activation to morphine compared to D2 mice (Belknap J. K. *et al.*, 1989; Phillips T. J. *et al.*, 1994a), and MALDR-2 mice had greater locomotor activation to both MOP-r agonist drugs compared to MAHDR-2 mice. However, these similarities did not extend to sensitivity to the analgesic effects of MOP drugs. It is not surprising that the selected lines did not resemble the inbred strains for all traits examined, since the lines were created to differ for a specific response and for those that share some genetic codetermination with that response. Genetic differences between inbred strains arise by chance, rather than by selective breeding; thus, not all phenotypic differences, even if under genetic control, would be expected to share common genetic regulation in a pair of inbred strains. Overall, these results suggest that some of the same D2-like genes that have a role in greater MA consumption also have a role in reduced sensitivity to the locomotor activating effects of opioids, but not in sensitivity to opioid analgesic effects.

It is possible that the MADR lines differ in level of opioid consumption. If MA and opioid consumption are genetically related and predicted from B6 and D2 strain differences, then opioid consumption should be greater in the low than high MADR line mice. We make this directional prediction because B6 mice have been shown to consume more morphine compared to D2 mice in two-bottle choice drinking procedures (Belknap J. K. *et al.*, 1993b; Berrettini W. H. *et al.*, 1994b). Although the possibility that there is no genetic relationship between MA and morphine intake must be considered, it is interesting to note that quantitative trait locus mapping results for MADR mice have identified a locus on proximal mouse chromosome 10

(unpublished) in the vicinity of the MOP-r gene, *Oprm1*, where morphine drinking has also been mapped (Berrettini W. H. *et al.*, 1994a; Berrettini W. H. *et al.*, 1994b; Ferraro T. N. *et al.*, 2005).

The current data support additional consideration of the MOP-r system for its role in differences in MA consumption between the high and low MADR line mice. To more directly investigate this, our future studies will examine MOP-r receptor levels and distribution and the effects of MOP-r agonists and antagonists on MA drinking. The greater sensitivity of MALDR-2 mice to some effects of MOP-r agonist drugs suggests that they may possess more of these receptors in brain regions relevant to these effects. Our findings suggest that greater MOP-r activity could result in lower MA intake and that MOP-r agonists could reduce MA intake.

## CHAPTER 3:

# Morphine intake and the effects of naltrexone and buprenorphine on the acquisition of methamphetamine intake

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*This manuscript has been published in the journal of Genes, Brains, and Behavior (February 2014; 13(2): 226-235.*

**Acknowledgements:** This work was supported by the Department of Veterans Affairs, and NIH grants T32 DA07262 and P50 DA018165. The authors would like to thank Harue Baba and Sue Burkhart-Kasch for assistance in the MA two-bottle choice experiments. The authors have no financial conflicts of interest to disclose.

## ABSTRACT

Some common genetic factors appear to influence risk for drug dependence across multiple drugs of abuse. In previous research, mice that were selectively bred for higher amounts of methamphetamine consumption, using a two-bottle choice methamphetamine drinking procedure, were found to be less sensitive to the locomotor stimulant effects of morphine and the more μ-opioid receptor selective agonist fentanyl, compared to mice that were bred for low methamphetamine consumption. This suggested that μ-opioid receptor mediated pathways may influence genetic risk for methamphetamine consumption. We hypothesized that these differences in opioid sensitivity would impact opioid intake in the methamphetamine drinking lines and that drugs with μ-opioid receptor activity would impact methamphetamine intake. Consumption of morphine was examined in 2, two-bottle choice studies, one that compared morphine to quinine consumption and another that used a saccharin fading procedure. Next, naltrexone (0, 0.5, 1, 2, 5, 10, 20 mg/kg), a μ-opioid receptor antagonist, and buprenorphine (0, 1, 2, or 4 mg/kg), a μ-opioid receptor partial agonist, were each examined for their effects on the acquisition of methamphetamine consumption. Low methamphetamine drinking mice consumed more morphine compared to high methamphetamine drinking mice. Naltrexone did not alter methamphetamine consumption in either selected line; however, buprenorphine reduced methamphetamine intake in the high methamphetamine drinking line. These data show that greater sensitivity to opioids is associated with greater opioid intake and indicate a need for investigation of drugs with μ-opioid receptor-specific agonist activity in genetically-determined differences in methamphetamine consumption.

## INTRODUCTION

Methamphetamine (MA) is a profoundly addictive psychostimulant (Panenka W. J. *et al.*, 2013). Though several pharmacological treatments to reduce MA use and dependence have been studied, effectiveness has been low (Karila L. *et al.*, 2010). Genetically-determined variation in mechanisms that contribute to MA use and dependence may inform treatment development. To study this, we used selectively bred MA high drinking (MAHDR) and MA low drinking (MALDR) mouse lines (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). MAHDR mice consume ~6 mg/kg MA in an 18-h period, while MALDR mice consume little to no MA (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011); MAHDR mice exhibit sensitivity to rewarding and reinforcing effects of MA, whereas MALDR mice are insensitive (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Shabani S. *et al.*, 2012a); MALDR mice exhibit high sensitivity to aversive effects of MA, whereas MAHDR mice exhibit insensitivity (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Shabani S. *et al.*, 2012b); and MALDR mice exhibit greater sensitivity to drugs with μ-opioid receptor (MOP-r) agonist activity, compared to MAHDR mice (Eastwood E. C. & Phillips T. J., 2012).

The latter results indicate that there is some common genetic influence on risk for MA intake and on sensitivity to opioid drugs and suggest that innate differences in opioid pathways could influence MA intake. They also suggest that greater sensitivity to MOP-r-mediated effects may serve a protective role against MA intake. To further examine opioid differences between MALDR and MAHDR mice, we asked whether the lines differ in morphine consumption. Data from the progenitor strains of these lines showed that greater MA intake was associated with reduced morphine intake (Belknap J. K. *et al.*, 1993a; Eastwood E. C. & Phillips T. J., 2012); thus, we anticipated that MALDR mice would consume more morphine than MAHDR mice. We additionally investigated whether the acquisition of MA intake could be altered by drugs with MOP-r activity. The MOP-r antagonist naltrexone (NTX) reduced MA intake in self-administering rhesus macaques and reduced the subjective effects of MA in human dependent users

(Jayaram-Lindstrom N. *et al.*, 2004; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b). However, the MOP-r partial agonist buprenorphine (BUP) has also shown efficacy in reducing human psychostimulant use (Kosten T. R. *et al.*, 1989a; Kosten T. R. *et al.*, 1989b; Mello N. K. *et al.*, 1989; Kosten T. R., 1992). Because reduced MA use has been found in human studies using both an agonist and antagonist, we chose to study both in our model of genetically high and low risk for MA intake. BUP was chosen over a full agonist because of its potential treatment efficacy in human populations and due to the higher abuse liability for full MOP-r agonists like morphine. The effects of MOP-r drugs on acquisition, rather than established MA drinking, were examined to study genetically-determined establishment of MA intake. Due to reduced inherent MOP-r regulated activity in MAHDR mice compared to MALDR mice, we predicted that BUP would simulate the MALDR phenotype and decrease the acquisition of MA drinking in MAHDR mice and that NTX might simulate the MAHDR phenotype and increase acquisition in MALDR mice.

## Materials and Methods

### Morphine drinking

The few morphine drinking studies in the literature have used two different procedures. We examined morphine drinking in the MADR lines using both procedures. Both involved the use of saccharin, and one used a quinine tastant control.

**Morphine-quinine drinking.** This study utilized a continuous access, two-bottle choice procedure (Table 3.1). Mice were tested in two cohorts (final N=8-14/sex/line) and were 85-110 days of age. Mice were isolate housed on day 1 and offered two, 25-ml tubes containing tap water for 2 days to acclimate to the drinking apparatus. They were then offered the choice between the two, 0.2% saccharin sweetened solutions listed in Table 3.1 for 4-day periods. In the second cohort of this study, the 0.7 mg/ml morphine concentration used in the first cohort was replaced with a higher 1 mg/ml morphine solution and the quinine concentration was adjusted to 0.55 mg/ml; results from the first cohort (explained below) led to this change.

Amount of fluid consumed was measured daily to the nearest 0.2 ml. Body weight data obtained every other day were used to determine amount of morphine, quinine and saccharin consumed in mg/kg. Total volume consumed (ml/kg) was also examined. The position of the morphine-containing tube, relative to the non-morphine tube, was switched every 2 days to control for possible side biases that may have been present for individual animals. To correct for tube leakage/evaporation, the volume lost from control tubes on cages devoid of mice were subtracted from each drinking tube.

**Table 3.1: Schedule and fluids offered during morphine vs quinine two-bottle choice study**

| Day | Tube 1 | Tube 2 |
|---|---|---|
| 1-2 | water | water |
| 3-6 | 0.2% saccharin + 0.3 mg/ml morphine | 0.2% saccharin + 0.2 mg/ml quinine |
| **Pass 1:** | | |
| 7-10 | 0.2% saccharin + 0.7 mg/ml morphine | 0.2% saccharin + 0.4 mg/ml quinine |
| **Pass 2:** | | |
| 7-10 | 0.2% saccharin + 1.0 mg/ml morphine | 0.2% saccharin + 0.55 mg/ml quinine |

**Morphine-saccharin drinking.** This procedure (Table 3.2) also used saccharin, but in a classic saccharin fading procedure like that used in previous studies of opiate (Forgie M. L. *et al.*, 1988; Belknap J. K. *et al.*, 1993a) and ethanol (Samson H. H., 1986; Weiss F. *et al.*, 1990) consumption to initially mask the bitter taste of these substances and make them more palatable. Mice were tested in one cohort (final N=9-13/sex/line) and were 82-104 days of age. The concentration of morphine was gradually increased in a fixed concentration of saccharin (0.2%) and then saccharin was gradually faded out. Throughout this procedure, one bottle contained unadulterated tap water. Mice were individually housed on day 1 of the study and tested as described for the morphine-quinine study.

**Table 3.2: Schedule and fluids offered during saccharin fading morphine two-bottle choice study**

| Day | Tube 1 | Tube 2 |
|---|---|---|
| 1-2 | water | water |
| 3-6 | water | 0.2% saccharin |
| 7-10 | water | 0.2% saccharin + 0.3mg/ml morphine |
| 11-14 | water | 0.2% saccharin + 0.5 mg/ml morphine |
| 15-18 | water | 0.2% saccharin + 0.7 mg/ml morphine |
| 19-22 | water | 0.07% saccharin + 0.5 mg/ml morphine |
| 23-26 | water | 0.02% saccharin + 0.5 mg/ml morphine |
| 27-30 | water | 0.5 mg/ml morphine |

**Effect of NTX on MA two-bottle choice drinking**

Two separate studies that used a 12-day procedure were conducted. In the first study, mice were 76-116 days old and data were collected in 3 cohorts (final N=6/sex/line/dose). A limited access drinking procedure was used to maximize intake by offering MA during the initial part of the dark phase of the light:dark cycle. Mice were placed on a reverse light:dark cycle (lights off at 0830 h and on at 2030 h), at least 2 weeks before the study began. Single housing and acclimation to 25-ml drinking tubes were as described for the morphine drinking studies. On days 3 and 4, mice received saline injections 30 min before dark phase onset to familiarize them with handling and injection. On day 5, saline or NTX (0.5, 1, or 2 mg/kg) was administered 30 min before dark phase onset. The doses of NTX were based on previous experiments measuring effects of NTX on ethanol consumption in our and other labs (Phillips T. J. *et al.*, 1997; Kamdar N. K. *et al.*, 2007). A water tube was replaced with one containing MA at lights off for a 6-h period. Effects of NTX on consumption of water and 20 and 40 mg/l MA solutions were examined. These are the concentrations that were used to create the MADR lines. Each concentration was offered for 4 days. Readings were taken every 2 h. At the end of the 6-h period, the MA tube was removed and the water tube left in place. Mice were weighed every other day and had *ad libitum* access to food.

Results from the first study, using low doses of NTX, indicated no significant effects on MA intake. Based on data showing effects of NTX doses of 0.5-40 mg/kg on locomotor activity and ethanol intake (Castellano C. & Puglisi-Allegra S., 1982; Kiianmaa K. *et al.*, 1983), we tested additional doses of 5, 10, and 20 mg/kg. All other study details were identical. Data were collected in 4 cohorts (final N=8/sex/line/dose and mice were 74-101 days old.

**Effect of BUP on MA two-bottle choice drinking**

All experimental details were identical to those used in the NTX-MA two-bottle choice drinking study, except that 10-ml drinking tubes were used (accuracy=0.1 ml). Mice received

injections of either saline or BUP (1, 2, or 4 mg/kg), 30 min before dark phase onset. Data were collected in 4 cohorts (final N=6/sex/line/dose) and mice were 67-94 days old.

**Statistical Analysis**

Repeated measures ANOVA, with morphine or MA concentration as the repeated factor, was used to analyze drug or quinine consumption in mg/kg and total volume consumed in ml/kg. Possible independent variables included sex, pretreatment drug dose (BUP or NTX), and selected line. Significant two-way interactions were resolved by simple main effects analysis and post-hoc mean comparisons were conducted when appropriate, using the Newman-Keuls test. Figures were created using Sigmaplot (Version 8.0; SPSS, Chicago, IL). The criterion for significance was set at $p \leq 0.05$.

## RESULTS

***Morphine-quinine drinking***

In the first cohort of mice, consumption of 0.3 and 0.7 mg/ml morphine vs quinine was examined. For morphine consumed (Fig. 3.1A), a significant concentration x line interaction was found ($F(1,42)=11.1$; $p<.01$). MALDR-2 mice consumed significantly more morphine than MAHDR-2 mice at the 0.7 ($p<.01$), but not 0.3, mg/ml concentration. A significant concentration x sex interaction was also identified ($F(1,40)=5.7$; $p<.05$), but data are shown for the sexes combined because this interaction was not dependent upon selected line. Both male and female mice showed increased morphine consumption with increasing concentration (both p values <0.001), but females consumed significantly more morphine than males ($p<.001$) only when it was offered as a 0.7 mg/ml solution (mean ± SEM: 118.0 ± 8.4 and 83.1 ± 6.6mg/kg for female and male, respectively).

For quinine consumption (Fig. 3.1B), a significant main effect of concentration was found ($F(1,42)=16.2$; $p<.001$); more quinine was consumed at the higher concentration, but there were no interactions of concentration with line or sex. There was a significant line x sex interaction ($F(1,40)=4.2$; $p<.05$). The line difference in quinine consumption was only significant in male

mice, with male MAHDR-2 mice consuming more quinine compared to male MALDR-2 mice ($p<.05$; mean ± SEM: 21.3 ± 4.4 and 7.9 ± 4.5 mg/kg for MAHDR-2 and MALDR-2, respectively).

On the first two water habituation days, there were no differences in total water consumed between the selected lines or sexes. Analysis of total volume consumed when the low and high concentrations of morphine were offered (Fig. 3.1C), did not identify any line or sex differences.

In the second cohort of mice, consumption of 0.3 and 1 mg/ml morphine vs quinine was measured. The higher concentration was included to examine whether the lines would continue to differ in morphine consumption, when a more concentrated solution was offered. For morphine consumed (Fig. 3.1D), there were significant effects of line ($F(1, 18)=6.6$; $p<.05$), sex ($F(1,18)=5.4$; $p<.05$), and concentration ($F(1,18)=9.0$; $p<.01$), but no interactions. MALDR-2 mice consumed more morphine than MAHDR-2 mice, female mice consumed more morphine compared to male mice, and more morphine was consumed when offered at the higher concentration.

For quinine consumption, there was a significant concentration x line interaction ($F(1,20)=4.6$; $p<.05$), but no effect of sex (Fig. 3.1E). Both MADR lines significantly increased quinine consumption as the concentration was increased (both p-values <.05); MAHDR-2 mice consumed significantly more quinine compared to MALDR-2 mice, when offered as a higher concentration ($p<.001$; mean ± SEM: 107.5 ± 15.3 and 50.6 ± 14.0 mg/kg for MAHDR-2 and MALDR-2, respectively). This higher intake in MAHDR-2 mice maintained a constant level of total fluid intake, as they drank relatively less from the morphine-containing tube.

Analysis of total volume consumed during the first two water habituation days did not identify any line or sex differences. Analysis of total volume consumed, when either the low or high concentrations of morphine were offered (Fig. 3.1F), identified a significant main effect of concentration ($F(1,20)=6.2$; $p<.05$), with somewhat reduced total volume consumed when the

higher concentrations of morphine and quinine were offered, but there were not significant effects of line or sex.

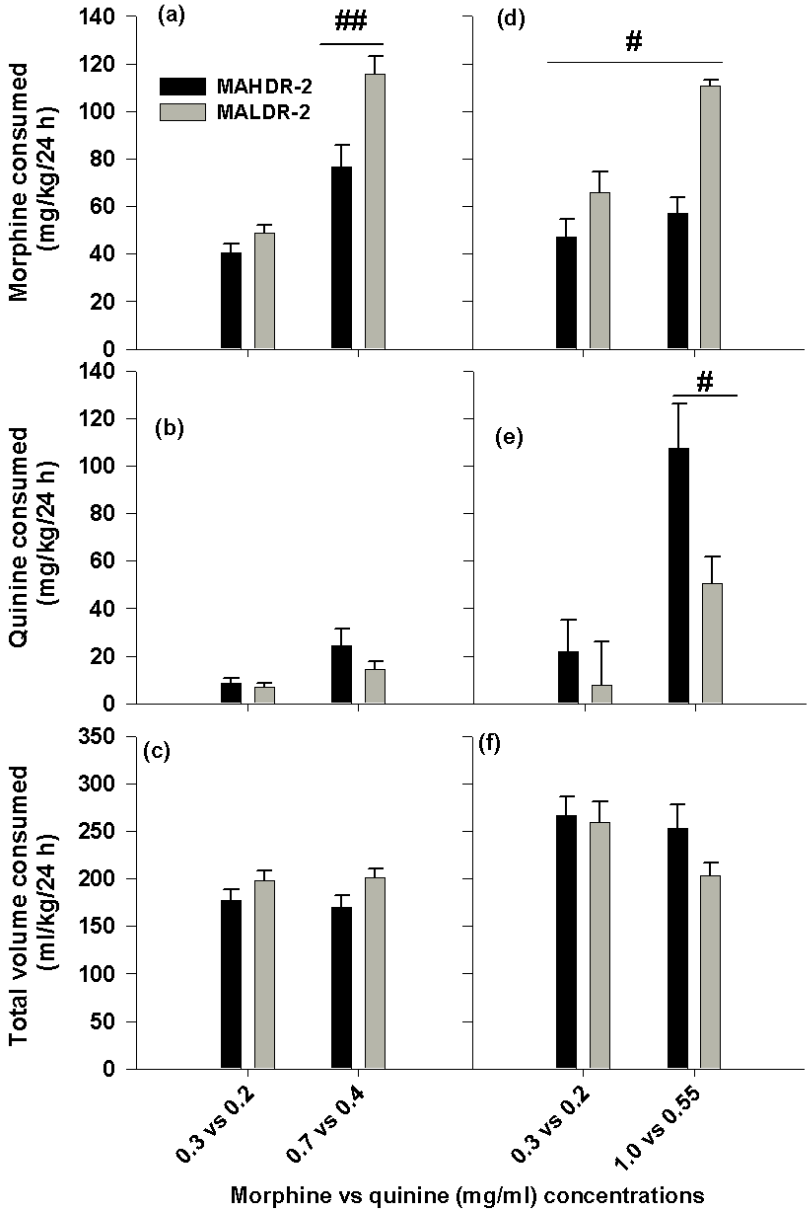


**Figure 3.1: Consumption of morphine and quinine in MADR mice, when offered as alternative solutions in a two-bottle choice study.** Shown in panel A, B and C are mean ± SEM morphine, quinine, and total volume consumed, for 0.3 and 0.7 mg/ml concentrations of morphine (in 0.2% saccharin) vs 0.2 and 0.4 mg/ml concentrations of quinine (in 0.2% saccharin) in a 24-h, two-bottle choice preference test. Panels D, E and F show similar data for 0.3 and 1.0 mg/ml morphine (in 0.2% saccharin) vs 0.2 and 0.55 mg/ml quinine (in 0.2% saccharin) concentrations, and total volume consumed. Each bar is a two-day average for days

2 and 4 of a 4-day drinking period. #p<.05, ##p<.01, for the line difference at the indicated concentration; +p<.05 for the main effect of line. Final N=8-14/sex/line.

***Morphine-saccharin drinking***

For sweetened and unsweetened morphine consumed in the absence of a quinine choice, a significant concentration x line interaction was found ($F(5,215)=2.6$; $p<.05$). The lines did not differ significantly in morphine consumption when the two lower concentrations were initially offered in saccharin. However, MALDR-2 mice consumed more morphine than MAHDR-2 mice at the highest concentration in saccharin and at all other concentrations as saccharin was faded out (Fig 3.2a). There was also a significant concentration x sex ($F(5,205)=6.5$; $p<.001$) interaction, with female mice consuming more morphine than males at all concentrations ($p<.05$ for all) except the last two, when the saccharin concentration was low or completely faded out. Because this difference was not dependent on line, data are shown for the sexes combined; however, mean ± SEM for the solutions shown along the x-axis in Fig 3.2 were 44.9 ± 5.8, 55.7 ± 8.9, 70.0 ± 16.2, 45.8 ± 11.1, 31.0 ± 8.2, and 24.7 ± 6.5 for the males, and 79.6 ± 4.8, 112.6 ± 7.5, 132.9 ± 13.7, 76 ± 49.4, 45.8 ± 6.9, and 28.1 ± 5.5 for the females).

The lines and sexes did not differ in total water intake during the first two water habituation days. However, analysis of total volume consumed during the morphine drinking phase of the study (Fig 3.2b) identified a significant concentration x line ($F(5,215)=6.1$; $p<.001$) interaction, but no effects of sex. MAHDR-2 and MALDR-2 mice did not differ in volume consumed for the first four types of solutions offered, but MAHDR-2 mice consumed greater total volume during the later phases (final two solution types; $ps < .01$).

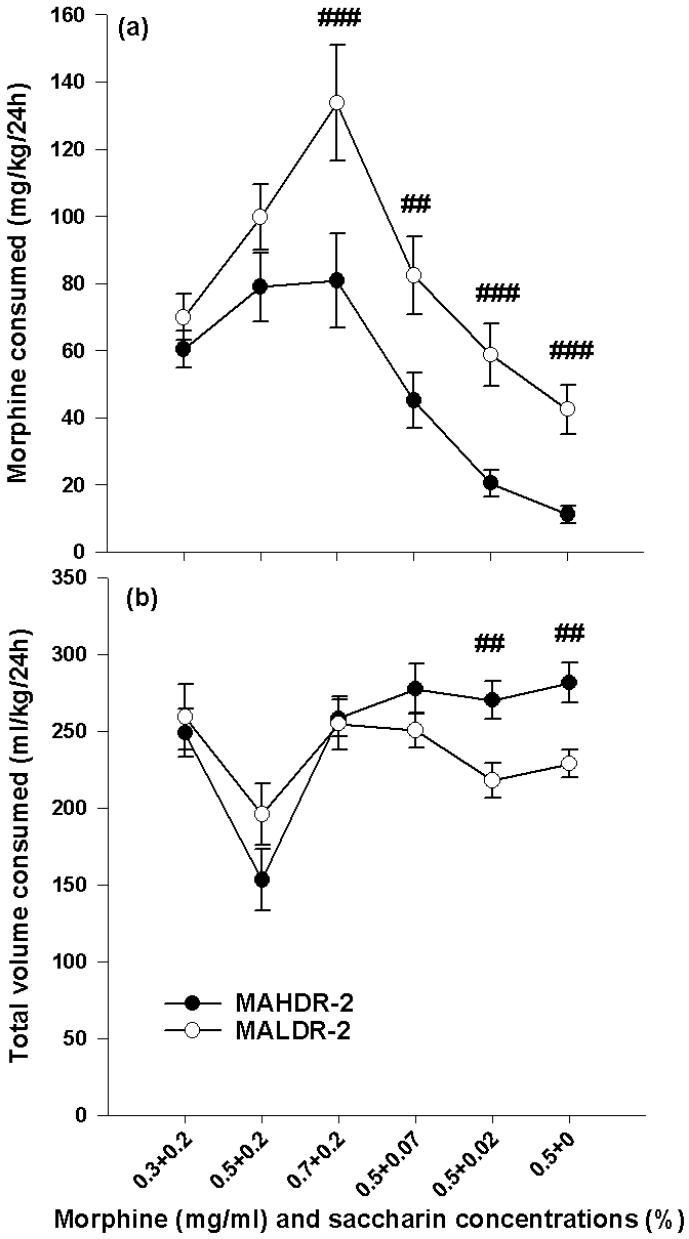


**Figure 3.2: Consumption of morphine by MADR mice in a saccharin fading study.** (A) shows mean ± SEM morphine consumed when offered at increasing concentrations in a fixed concentration of saccharin and then in a fixed concentration at decreasing saccharin concentrations. (B) shows mean ± SEM total volume consumed. Each data point is a two-day average for days 2 and 4 of a 4-day drinking period. #p<.05, ##p<.01, ###p<.001 for the line difference at the indicated concentration. Final N=9-13/sex/line.

**Effect of NTX on MA two-bottle choice drinking**

*Lower NTX doses.* Lower doses of NTX had no significant effect on MA consumed, when data were examined for the entire 6-h access period (Fig. 3.3A and B). When data for each 2-h block were included in an overall analysis, there were no significant effects of NTX associated with different time periods (data not shown). There was a significant line x sex interaction ($F(1,63)=4.6$; $p<.05$) for consumption of the 20 mg/l MA solution; as has been found previously (e.g., Shabani et al., 2011), there was comparable avoidance of MA by male and female MALDR-2 mice, whereas female MAHDR-2 mice consumed more MA than male MAHDR-2 mice (mean ± SEM: 1.36 ± 0.07 and 0.97 ± 0.09 mg/kg for female and male MAHDR-2 mice, respectively). Results were similar for the 40 mg/l MA concentration, showing a significant line x sex interaction ($F(1,63)=10.0$; $p<.01$), and greater intake in female compared to male MAHDR-2 mice ($p<.001$; mean ± SEM: 2.8 ± 0.2 and 1.9±0.2mg/kg for female and male MAHDR-2 mice, respectively). As shown in Fig. 3.3C, MAHDR-2 mice consumed greater total volume compared to MALDR-2 mice at both concentrations of MA ($F(1,71)=13.2$; $p<.001$ and $F(1,71)=19.7$; $p<.001$ for the period when 20 and 40 mg/l MA was offered, respectively). Female mice consumed more total volume compared to males ($F(1,63)=23.7$; $p<.001$ and $F(1,63)=23.0$; $p<.001$ for 20 and 40 mg/l phases, respectively), but this sex effect did not interact with line.

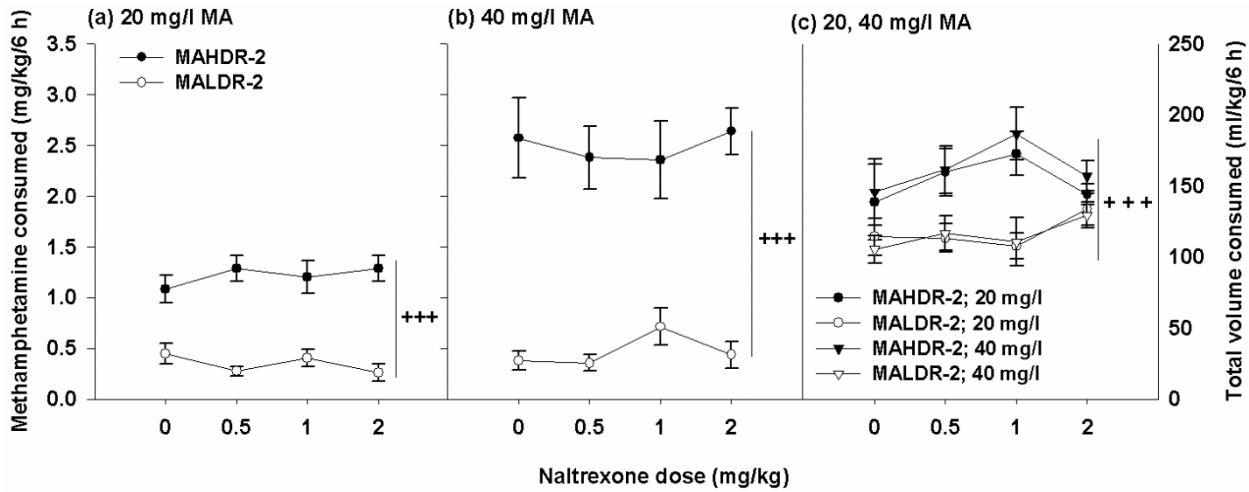


**Figure 3.3: Pretreatment with naltrexone does not alter MA drinking in MADR mice.** (A) and (B) show mean ± SEM 20 mg/l and 40 mg/l MA consumed, when offered vs water in a 6-h limited access procedure during the beginning of the dark phase of the light : dark cycle. (C) shows mean ± SEM total volume intake. Mice were treated with saline or one of several doses of naltrexone 30 min prior to MA drinking tube access. Each data point is a two-day average for days 2 and 4 of a 4-day drinking period. +++p<.001 for the main effect of line. Final N=6/sex/line/dose.

*Higher NTX doses*. Higher doses of NTX had no significant effect on MA consumed, when data were examined for the entire 6-h MA access period (Fig. 3.4A and B). When data for each 2-h block were included in an overall analysis, there were no significant effects of NTX associated with different time periods (data not shown). There was a significant main effect of line ($F(1,117)=76.8$; $p<.001$) for consumption of the 20 mg/l MA concentration, with MAHDR-2 mice consuming more MA than MALDR-2 mice. There were no interactions with sex or NTX dose. Results were similar for the 40 mg/l MA concentration; there was only a significant main effect of line ($F(1,117)=84.1$; $p<.001$). As shown in Fig. 3.4C, MAHDR-2 mice again consumed greater total volume than MALDR-2 mice at both concentrations of MA ($F(1,109)=14.2$; $p<.001$ and $F(1,109)=10.4$; $p<.001$ for the period when 20 and 40 mg/l MA was offered, respectively). Female mice consumed more total volume compared to males ($F(1,109)=6.9$; $p<.01$ and $F(1,109)=12.2$; $p<.01$ for 20 and 40 mg/l phases, respectively), but this sex effect did not interact with line.

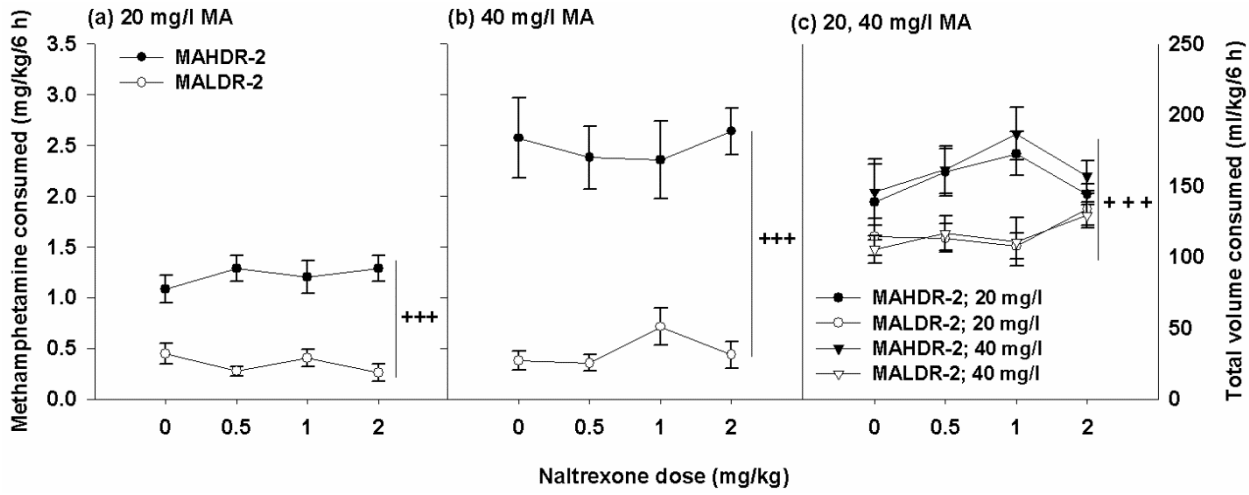


**Figure 3.4: Pretreatment with higher doses of naltrexone does not alter MA drinking in MADR mice.** (A) and (B) show mean ± SEM 20 mg/l and 40 mg/l MA consumed, when offered vs water in a 6-h limited access procedure during the beginning of the dark phase of the light : dark cycle. (C) shows mean ± SEM total volume intake. Mice were treated with saline or one of several doses of naltrexone 30 min prior to MA drinking tube access. Each data point is a two-day average for days 2 and 4 of a 4-day drinking period. ++p<.01; +++p<.001 for the main effect of line. Final N=8/sex/line/dose.

**Effect of BUP on MA two-bottle choice drinking**

BUP had dose- and time-dependent effects on MA intake. For the 20 and 40 mg/l concentrations, when data for each 2-h block were included in an overall analysis, there were significant time x line ($F(2,198)=5.3$, $p<0.01$ and $F(2,198)=4.7$, $p<0.01$ for 20 and 40 mg/l, respectively) and significant time x dose ($F(6,198)=3.1$; $p<0.01$ and $F(6,198)=2.9$, $p<0.01$ for 20 and 40 mg/l, respectively) interactions. We next examined effects within each 2-h period, both because of the significant time interactions and because BUP is known to have a half-life of about 3 h (Yu et al., 2006). For the 20 mg/l concentration of MA, there was a significant line x BUP dose interaction for the first two, 2-h time periods ($F(3,99)=2.5$; $p=0.05$ and $F(3,99)=3.6$; $p<.05$), but not during the last 2-h period. During the first 2 h (Fig. 3.5A), there was a significant effect of BUP dose only within the MAHDR-2 line ($p<.01$), and post-hoc tests indicated that the 1 and 2 mg/kg doses significantly reduced MA intake, compared to saline. Treatment with these doses of BUP eliminated the significant line difference in MA intake. During the second 2-h period (Fig. 3.5D), there was a significant effect of BUP dose, again only in MAHDR-2 mice ($p<.05$), and all doses significantly reduced MA intake. The significant line difference in MA intake was eliminated by all 3 BUP doses. During the last 2-h period (Fig. 3.5G), only a significant main effect of line was found for MA intake ($F(1,91)=42.8$; $p<.001$), with no significant effect of BUP treatment.

BUP was less effective in reducing intake of the 40 mg/l concentration of MA. There was a significant line x BUP dose interaction for the first 2-h time period ($F(3,99)=4.2$; $p<.01$), but not during subsequent time periods. For the first 2-h period (Fig.3.5B), there was a significant effect of dose only within the MAHDR-2 line ($p<.01$); the 1 and 2 mg/kg doses of BUP reduced MA intake, compared to saline. The line difference for MA intake was reduced by these BUP doses, but not eliminated. For the second and third 2-h periods (Fig. 3.5E and H), only the main effect of line was significant, with MAHDR-2 mice consuming significantly more MA compared to MALDR-2 mice ($F(1,99)=105.6$; $p<.001$and $F(1,99)=61.7$; $p<.001$, respectively).

For total volume consumed during the BUP study, there were significant BUP effects only in MAHDR-2 mice. When the 20 mg/l concentration of MA was offered, there was a significant line x BUP dose interaction for the first two, 2-h periods ($F(3,99)=5.2$; $p<.01$ and $F(3,99)=2.9$; $p<.05$). During the first 2 h, there was a significant effect of BUP dose within the MAHDR-2 mice ($p<.001$); the 1 mg/kg BUP dose significantly reduced total volume intake, compared to saline (Fig. 3.5C). During the second 2 h, the effect of dose within the MAHDR-2 line was significant ($p<.05$); 2 mg/kg BUP reduced total volume intake. When the 40 mg/l concentration of MA was offered, there was no significant BUP effect on total volume during the first or last 2-h periods (Fig. 3.5F). During the third 2 h, there was a significant line by BUP dose interaction ($F(3,99)=3.1$; $p<.05$), but this was associated with MALDR-2 mice consuming greater total volume than MAHDR-2 mice for the 1 mg/kg treatment groups ($p<.05$), rather than with significant BUP treatment effects in either of the MADR lines (Fig. 3.5I).

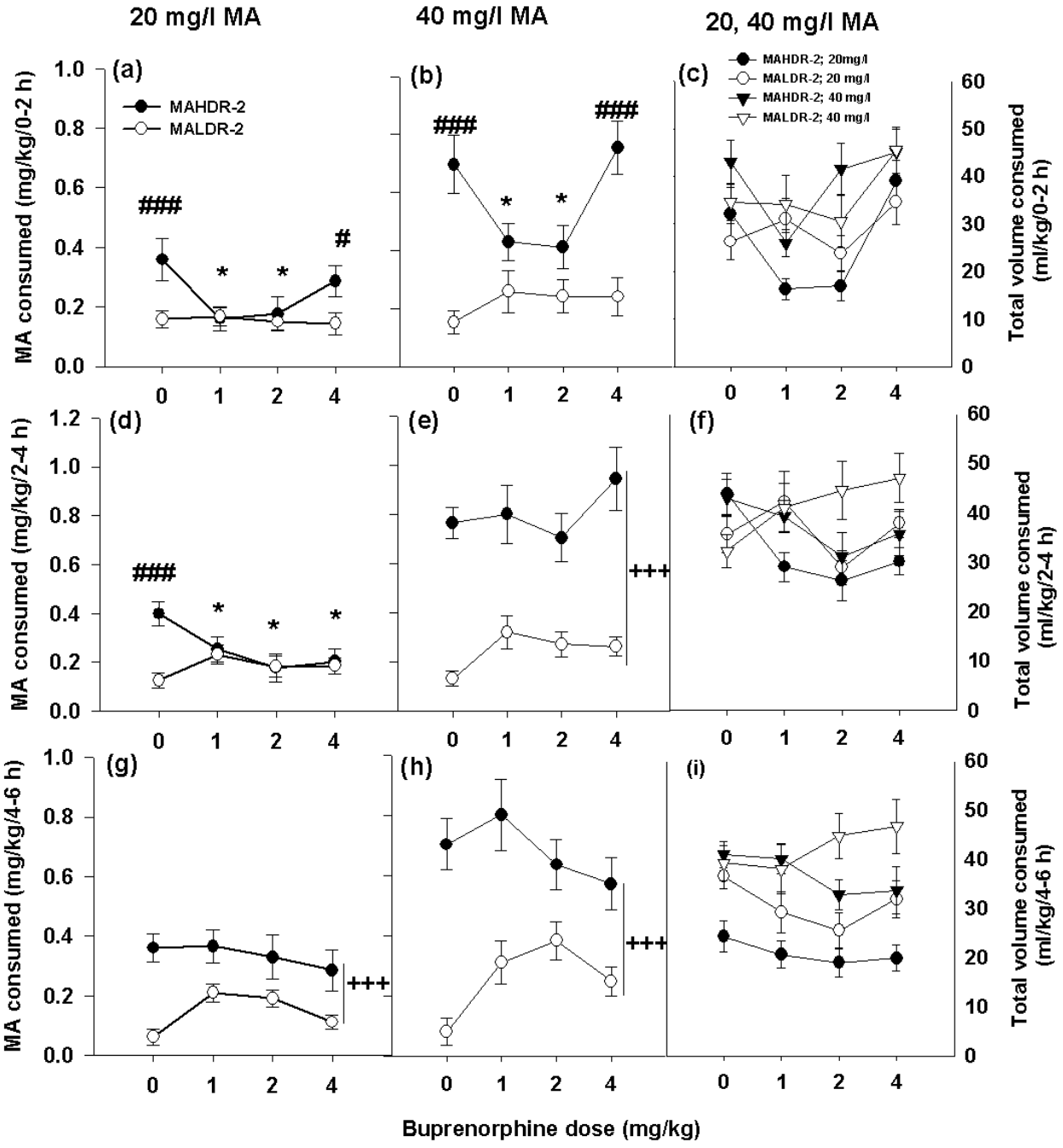


**Figure 3.5: Lower doses of BUP reduce MA intake.** Shown are mean ± SEM 20 and 40 mg/l MA consumption and total volume intake for the first (A-C), second (D-F) and third (G-I) 2-h periods of a 6-h MA vs water access period. The legend in panel a applies to panels A, B, D, E, G and H; the legend in panel c applies to panels C, F and I. Mice were treated with saline or one of several doses of BUP 30 min prior to the 6-h access period during the dark phase of the light

: dark cycle. Each data point is a two-day average for days 2 and 4 of a 4-day drinking period. #p<.05, ##p<.01, ###p<.001, for the line difference at the indicated dose. *p<.05 for the difference between the saline and BUP mean at the indicated dose. +++p<.001 for the main effect of line. Final N=6/sex/line/dose.

## DISCUSSION

Our data indicate that selective breeding for MA consumption has altered voluntary intake of solutions containing morphine. We previously showed that MALDR mice have greater sensitivity to both a non-selective and selective MOP-r agonist (Eastwood E. C. & Phillips T. J., 2012). Therefore, MOP-r mediated pathways may contribute to both the difference in morphine intake and to genetically-determined susceptibility to MA drinking. Lower doses of BUP interfered with the acquisition of MA intake in mice of the MAHDR selected line during the first 4 h of a 6-h limited access procedure, whereas NTX doses from 0.5 to 20 mg/kg did not significantly alter MA intake in either selected line.

The effect of BUP on MA intake could be due to its MOP-r partial agonist activity; at lower doses, BUP activates the MOP-r, but at higher doses it is known to act as a MOP-r antagonist and may also act via non-classical opioid mechanisms (Pick C. G. *et al.*, 1997; Ciccocioppo R. *et al.*, 2007). BUP has been shown to increase ethanol intake at lower doses and reduce it at higher doses (Ciccocioppo R. *et al.*, 2007). Full MOP-r agonist drugs have also been found to increase ethanol intake (Hubbell C. L. *et al.*, 1986; Hubbell C. L. *et al.*, 1993; Zhang M. & Kelley A. E., 2002). The higher dose effects of BUP are consistent with opioid receptor antagonists being effective at reducing ethanol intake (Altshuler H. L. *et al.*, 1980; Myers R. D. & Critcher E. C., 1982; Critcher E. C. *et al.*, 1983; Phillips T. J. *et al.*, 1997; Ciccocioppo R. *et al.*, 2007). However, BUP is known to act as a partial agonist at μ, δ, and κ opioid receptors (Ide S. *et al.*, 2004b) and further investigation is required to determine whether the effects on MA intake are specifically MOP-r mediated. Current studies are examining the effect of the more specific MOP-r agonist drug, fentanyl on MA intake, and data are being

collected with better time resolution by using a lickometer system. Future studies are also planned to examine MA drinking in MOP-r knockout vs wildtype mice. The current MA concentration-dependent effects of BUP could be explained by a BUP-induced right-ward shift in the MA dose-response curve. Similar findings have been demonstrated in D2 mice treated with BUP prior to a cocaine self-administration session, where mice were administered various concentrations of cocaine (Kuzmin A. V. *et al.*, 2000).

Unlike our results for MA drinking, others have shown that MA-induced locomotor activation and sensitization were attenuated by treatment with MOP-r antagonists and in MOP-r knockout mice (Jones D. N. & Holtzman S. G., 1994; Chiu C. T. *et al.*, 2005; Shen X. *et al.*, 2010). However, the MA drinking lines show comparable locomotor stimulation and sensitization to most doses of MA (Shabani S. *et al.*, 2011), suggesting that, in these lines, MA drinking and these motor responses are not influenced by common genetic mechanisms. Therefore, specific aspects of opioid pathways that are involved in genetic sensitivity to MA stimulation and sensitization vs risk for MA drinking may be largely different.

Supporting MOP-r involvement in human MA intake, a linkage disequilibrium block and polymorphisms in the MOP-r gene, *OPRM1* have been associated with MA-induced psychosis and dependence (Ide S. *et al.*, 2004a; Ide S. *et al.*, 2006; Deb I. *et al.*, 2010); however, see Heinzerling et al. 2012). Our lab has completed quantitative trait locus (QTL) analysis in both sets of MADR lines and has identified a QTL on proximal mouse chromosome 10 that accounts for approximately 50% of the genetic variance in MA consumption between the selected lines (Belknap et al., 2013; in press). Though many other genes reside in the identified interval on chromosome 10, the MOP-r gene is in that interval and MA naïve MALDR line mice have greater expression of *Oprm1* in medial prefrontal cortex (PFC) tissue compared to MAHDR line mice, but not in nucleus accumbens (NAc) or ventral tegmental area (VTA) tissue. The PFC sends glutamate projections to the NAc and VTA, which can modulate the amount of dopamine present in the NAc (Carr D. B. *et al.*, 1999). Local application of MOP-r agonists in the mPFC

reduced glutamate-induced PFC firing and resulted in a reduction in NAcc dopamine level (Sesack S. R. & Bunney B. S., 1989; Sesack S. R. & Pickel V. M., 1992b, 1992a; Giacchino J. L. & Henriksen S. J., 1998). Low doses of BUP have been shown to decrease MA-induced increases in dopamine in the NAc (Pereira F. C. *et al.*, 2011). Administration of psychostimulants has been shown to increase endogenous opioid neurotransmission in rats (Olive M. F. *et al.*, 2001; Roth-Deri I. *et al.*, 2003), but to the best of our knowledge, this has not been investigated in mice. Further, MOP-r knockout mice showed decreased dopamine and dopamine metabolite levels in the striatum following MA administration compared to control mice, suggesting a modulatory role of MOP-rs on MA-induced dopamine release (Lan K. C. *et al.*, 2008). It is possible that a difference in the expression of MOP-r between the MADR lines in the PFC could result in decreased dopamine levels in the NAc of MALDR mice compared to MAHDR mice when MA is administered. Less dopamine in the NAc would be expected to correspond with decreased reinforcement from MA in MALDR mice. Our QTL findings are being followed up with further narrowing of the relevant genetic region, which will be followed by more detailed analysis of remaining candidates.

A negative genetic correlation was found between MA and morphine consumption, for both a study that included a quinine choice and one that used saccharin fading. MALDR line mice consumed more morphine than MAHDR mice. The morphine vs quinine procedure (Berrettini W. H. *et al.*, 1994b) was chosen, because it was used to map a morphine consumption QTL to proximal chromosome 10 (Berrettini W. H. *et al.*, 1994a; Berrettini W. H. *et al.*, 1994b) in the same region as our MA drinking QTL. The saccharin fading procedure was performed to determine if the line difference in morphine consumption would persist in the absence of the addition of a sweetener and allowed the direct comparison of morphine to water consumption. In the morphine vs quinine drinking procedure, consumption of quinine mirrored the selected line difference for morphine consumption, so that total fluid intake was similar for the two selected lines (i.e., the one that consumed more of the morphine-containing solution

consumed less of the quinine solution and vice versa, so that total fluid intake was largely comparable). The motivation of the MAHDR line to consume more quinine may have been to avoid the morphine-containing solutions. We have noted previously that the selected lines, which were created from an F2 cross of the B6 and D2 inbred strains, resemble their progenitor strains on several, but not all, traits (Eastwood E. C. & Phillips T. J., 2012). Similar to the D2 strain, the MAHDR line consumed lower amounts of morphine and higher amounts of quinine, whereas similar to the B6 strain, the MALDR line consumed higher amounts of morphine and lower amounts of quinine (Belknap J. K. *et al.*, 1993a; Blizard D. A. *et al.*, 1999). However, Belknap et al. (1993) and Blizard et al. (1999), found that B6 mice consumed more sucrose and saccharin compared to D2 mice, while we have not seen any differences between the MADR lines when offered saccharin or quinine vs water (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011), suggesting that the selected lines do not recapitulate all differences seen between the inbred strains.

MAHDR mice consumed a greater total volume of fluid compared to MALDR mice in some, but not all, studies. This difference was not seen in the morphine vs quinine drinking study, but it was in the later phases of the morphine vs water study and in the MA vs water 6-h limited access studies. On the baseline water days prior to each study, the MADR lines did not differ in total volume consumed, suggesting that the volume differences are associated with aspects of the drinking studies. Similarly the MAHDR lines consumed approximately 0.5 ml more total volume in the 18-h MA vs water drinking selection trait procedure (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). Because no differences were found in total volume on baseline water days, it is unlikely that heightened drinking in general explains the line differences observed in the morphine and MA drinking procedures. However, greater MA consumption in MAHDR mice could lead to behavioral activation and increased fluid consumption. That said, the lines did not differ in total volume in the BUP-MA drinking procedure and the MAHDR line, which has heightened drinking and is more likely to drink from

the MA tube, reduced its MA but not water intake following BUP treatment. This finding suggests that BUP had specific effects on MA intake and is consistent with results of others showing no reductions in water intake in rodents following treatment with BUP (Liles J. H. & Flecknell P. A., 1992; Ciccocioppo R. *et al.*, 2007; Tubbs J. T. *et al.*, 2011).

We had predicted that MA intake might be increased by NTX pretreatment in MALDR mice. Theoretically, this treatment could block the increased MA-induced opioid signaling in the PFC (discussed above) and increase MA-induced reinforcement. A large range of NTX doses was tested, with no significant effect. However, because MALDR mice show strong MA avoidance and conditioned aversion (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Shabani S. *et al.*, 2012b) increases in intake may be hard to achieve. This genetically-determined high sensitivity to the aversive effects of MA likely involves a different mechanism(s) than sensitivity to reinforcing effects and may be an extremely effective protective factor against MA use.

In summary, our current data show that selective breeding for MA consumption has altered genes that influence opioid drug consumption and sensitivity. Further, our data support a role for opioid mechanisms in MA intake. Due to mixed effects for the partial MOP-r agonist, we are currently investigating the effects of the MOP-r specific agonist, fentanyl. MOP-r agonists pose the risk of abuse liability. However, in the current genetic model, high genetic preference for MA is associated with low preference for morphine, suggesting that the risk would be reduced in individuals with a genetic propensity to consume MA. Future studies will examine the effectiveness of MOP-r agonists on established MA intake.

**CHAPTER 4:**

# Methamphetamine drinking microstructure in mice bred to drink high or low amounts of methamphetamine

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*This manuscript has been published in the journal of Behavioral Brain Research (June 20, 2014 272: 111-120).*

**Acknowledgements:** ECE developed hypotheses, designed the studies, analyzed and interpreted the data, and wrote the manuscript. AMA consulted on data analysis and interpretation. TJP co-developed the hypotheses, assisted with study design, assisted with data analysis approach and interpretation, and edited the manuscript. This work was supported by the Department of Veterans Affairs, and NIH grants T32 DA07262, P50 DA018165, and R24 AA020245.

## ABSTRACT

Genetic factors likely influence individual sensitivity to positive and negative effects of methamphetamine (MA) and risk for MA dependence. Genetic influence on MA consumption has been confirmed by selectively breeding mouse lines to consume high (MAHDR) or low (MALDR) amounts of MA, using a two-bottle choice MA drinking (MADR) procedure. Here, we employed a lickometer system to characterize the microstructure of MA (20, 40, and 80 mg/l) and water intake in MAHDR and MALDR mice in 4-h limited access sessions, during the initial 4 hours of the dark phase of their 12:12 h light:dark cycle. Licks at one-minute intervals and total volume consumed were recorded, and bout analysis was performed. MAHDR and MALDR mice consumed similar amounts of MA in mg/kg on the first day of access, but MAHDR mice consumed significantly more MA than MALDR mice during all subsequent sessions. The higher MA intake of MAHDR mice was associated with a larger number of MA bouts, longer bout duration, shorter interbout interval, and shorter latency to the first bout. In a separate 4-h limited access MA drinking study, MALDR and MAHDR mice had similar blood MA levels on the first day MA was offered, but MAHDR mice had higher blood MA levels on all subsequent days, which corresponded with MA intake. These data provide insight into the microstructure of MA intake in an animal model of differential genetic risk for MA consumption, which may be pertinent to MA use patterns relevant to genetic risk for MA dependence.

## INTRODUCTION

Genetic factors may influence who is and is not at risk for developing a pattern of methamphetamine (MA) use leading to dependence. Several genetic variants in human populations have been identified and associated with MA abuse, dependence, and psychosis (Bousman C. A. *et al.*, 2009). We have examined the heritability of MA drinking (MADR) in mouse lines that were selectively bred for oral consumption of either high (MAHDR) or low (MALDR) amounts of MA (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). Selective breeding produced MAHDR lines that consume approximately 6 mg/kg of MA during an 18-h MA access period, compared to 0.5 mg/kg MA intake in MALDR mice (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). Calculated heritability was 0.34 in the replicate 1 set of lines and 0.35 in replicate 2, indicating that ~35% of the variance in intake could be attributed to heritable genetic factors. In addition to higher consumption of the drug, MAHDR mice are more sensitive to the conditioned rewarding and reinforcing effects of MA, whereas MALDR mice are more sensitive to the aversive effects of MA (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2012b). The focus of the present study is on patterns of MA intake that may be informative with regard to genetic risk for further use.

Although we have examined the motivational drive for MA intake in our genetic model of high and low MA intake, we have not examined patterns of consumption during the time that MA drinking is established. Initial experiences are critical to further use and microstructural analysis of MA and water intake during this time period would provide information about the impact of differential genetic risk for intake on the acquisition of MA consumption. In previous work, MADR mice trained to perform an operant response to gain access to either a saccharin sweetened MA- or non-MA-containing tube did not differ in the amount of MA consumed during the first trial that it was offered. However, MALDR mice reduced their consumption during the next trial and the selected lines differed in MA consumption during all subsequent trials (Shabani S. *et al.*, 2012a). These data suggest that the MALDR line mice reduce their intake

after experiencing pharmacological effects of MA that they perceive as aversive, rather than in response to taste or some other peripheral factor. Our previously published data, examining both taste factors and sensitivity to the aversive effects of MA, support this conclusion (Shabani et al., 2011; 2012b; Wheeler et al., 2009).

The microstructure of MA and water consumption was examined during 4-h limited access sessions using a lickometer system. The lickometer system provides precise time resolution of drinking behavior by continuously recording each lick of the sipper tube. By grouping these licks into bouts, several drinking measures can be obtained including number of drinking bouts, time between bouts, size of bout, and bout duration. Microstructural data using lickometer and similar systems have been commonly generated for ethanol and sucrose drinking (Samson H. H. *et al.*, 1988; Cannon D. S. *et al.*, 1994; Samson H. *et al.*, 1996; Samson H. H., 2000; Ford M. M. *et al.*, 2005; Rhodes J. S. *et al.*, 2007; Ford M. M. *et al.*, 2009; Pastor R. *et al.*, 2010; Barkley-Levenson A. M. & Crabbe J. C., 2012). In a separate study, we examined MA blood levels at time points that corresponded with the lickometer procedure. We hypothesized that similar to the operant oral self-administration data, the MADR lines would not differ in amount of MA consumed on the first day of MA access, but would diverge in amount consumed and in structural components (e.g., number and size of drinking bouts) of consumption from the MA-containing bottle, with subsequent access. We anticipated that blood MA levels would correspond

## Materials and Methods

*Drinking pattern analysis*

Fluid intake was measured in 24, custom-made acrylic plastic lickometer chambers (17.8 x 10.2 x 10.2 cm) that have been used in our previous studies (Sharpe A. L. & Phillips T. J., 2009; Pastor R. *et al.*, 2010). The lickometer device was manufactured by MED Associates, Inc. (St Albans, VT). Each test chamber had a stainless steel wire grid floor (VWR; Tualatin, OR) and two small holes located in the back wall through which two metal sipper tubes were

introduced. Tubes were secured to the chamber wall to reduce the potential for displacement by the mice and thus, reduce the recording of false intake volumes. A hinged acrylic plastic lid with ventilation holes covered each chamber. Stainless steel sippers (Anacore, Bellmore, NY) were attached to polystyrene serological pipettes (10 ml volume; VWR) to create drinking tubes. The pipettes were trimmed to a 6 ml capacity to allow them to fit properly behind the lickometer chambers. Tube volumes (0.1 ml accuracy) were recorded at the beginning and end of each 4-h drinking session.

The wire floor of the chamber and the metal sipper tubes form open electrical circuits connected to the lickometer device. A circuit is closed when an animal stands on the metal floor and makes contact by licking a sipper tube. A software program (MED-PC IV; MED Associates, Inc.) was used to automatically record cumulative sipper contacts. Individual animal cumulative lick records (total number of licks) were extracted using Soft CR version 4 (MED Associates, Inc.), and appetitive (latency to first bout) and consummatory (bout frequency, bout size, bout duration, interbout interval, bout lick rate) variables were extracted from the cumulative records using a custom data analysis program written for the online software R project for Statistical Computing (http://www.r-project.org). No previous data of this nature have been collected for MA, but based on multiple previous studies examining patterns of ethanol and sucrose drinking, a bout was defined as a series of at least 20 licks with less than 1 minute separating each lick (Ford M. M. *et al.*, 2005; Ford M. M. *et al.*, 2009; Pastor R. *et al.*, 2010; Barkley-Levenson A. M. & Crabbe J. C., 2012).

*MA and water intake*

Data were collected in 2 equal size cohorts of mice. A 4-h limited access drinking procedure was used that was initiated at the beginning of the dark phase. This period was chosen as a time when consumption was expected to be relatively high, compared to other times within the 24-h day, similar to the approach that has been used to examine binge-like ethanol drinking (Rhodes J. S. *et al.*, 2005; Moore E. M. *et al.*, 2007; Rhodes J. S. *et al.*, 2007).

Mice were acclimated to single housing for 2 days before the drinking procedure began. On study day 1, at lights out, mice were placed into individual lickometer chambers and two tubes, both containing tap water, were extended into the cage to allow for acclimation to the sipper tubes and collection of baseline water only data. This was repeated on day 2. On days 3-14, mice were offered a tap water tube and a tube containing 20, 40, and then 80 mg/l MA in tap water, with each MA concentration provided for 4 consecutive days. After each session, mice were returned to their home cages. Mice were weighed every other day and had *ad libitum* access to food and water in both the lickometer chambers and their home cages. Drug and water tube sides were randomized across subjects.

*Blood MA levels*

To examine blood MA levels resulting from MA consumption, a separate limited access drinking study was performed. Procedures were identical to those used for the lickometer study, except that the lickometer apparatus was not used and the drinking of individual mice was measured in their home cages. This allowed volumetric readings to be taken at multiple time points, which may have altered the drinking patterns of mice in a lickometer study. Mice were assigned to one of 3 groups based on when 20 µl lateral tail vein blood samples were collected. On days 3, 6, 10, and 14, a 20 µl blood sample was collected from the lateral tail vein of mice at either 2 h into the 4-h session or immediately after the 4-h session, depending on group assignment. Group 1 mice had blood samples taken on the first day of MA access (day 3 of the study), and immediately after the 4-h session on the final day of access to 20 and 80 mg/l MA (days 6 and 14). Groups 2 and 3 had blood samples taken on the final day of access to each MA concentration (days 6, 10, and 14) at either 2 h into the session or at the end of the 4-h session. Each sample was placed into a microcentrifuge tube that contained 80 µl of EIA buffer provided by Neogen (Lexington, KY) and MA levels from blood samples were assessed using the Neogen amphetamine group enzyme-linked immunosorbent assay (ELISA) kit. Samples were read with a Bio-Rad Benchmark Plus microplate spectrophotometer (Hercules,

CA) equipped with a 450 nm filter. MA concentrations were determined using a calibration curve.

*Data Analysis*

All statistical analyses were conducted using Statistica version 6.1 software (StatSoft, Inc., Tulsa, OK). Repeated measures ANOVA, with selected line as the between-groups factor and MA concentration and time within the 4-h sessions as repeated factors, was used to analyze mg/kg MA consumed, ml/kg of total fluid consumed, cumulative licks, and drinking bout parameters. For some analyses, day within MA concentration was also used as a repeated measure. For analysis of the blood MA data, selected line and sampling time group were used as between-groups factors, with day of sample as a repeated measure. Significant two-way interactions were resolved using simple main effect analyses and post hoc mean comparisons were performed when appropriate, using the Newman-Keuls post hoc test. The criterion for significance was set at $p \leq 0.05$. Figures were created using Sigmaplot (Version 8.0; SPSS, Chicago, IL). Data from 9 mice (4 MALDR-2 and 5 MAHDR-2) were excluded due to technical difficulties with the lickometer equipment and interface.

## RESULTS

### MA and total volume consumed during the 4-h drinking session

Fig. 4.1A shows MA consumed during the 4-h drinking sessions on days 6, 10, and 14, which were the final days that each concentration was offered. Data on other days were also examined and some of those results are presented below. These data are presented because they provide a simple summary of findings for a period after maximal access and acclimation to each concentration of MA. There was a significant concentration x line interaction ($F[2,60]=6.6$; $p<0.01$). As expected, MAHDR-2 mice consumed more MA than MALDR-2 mice at every concentration. In addition, MAHDR-2 mice significantly escalated their MA intake as the concentration was increased ($p<0.001$, for the comparison between MA intake at 80 mg/l and 20 mg/l MA concentrations), whereas MALDR-2 mice showed no change in their low levels of

intake. Fig. 4.1B shows water consumed during the same 4-h periods. There was a main effect of line ($F[1,30]=14.2$; $p<0.001$), but no effect of the MA concentration offered during the water access period, nor interaction of these two factors. MALDR-2 mice consumed more water compared to MAHDR-2 mice. Fig. 4.1C shows total volume consumed in ml/kg. The MADR-2 lines did not differ in total fluid intake, nor were there MA concentration-dependent effects.

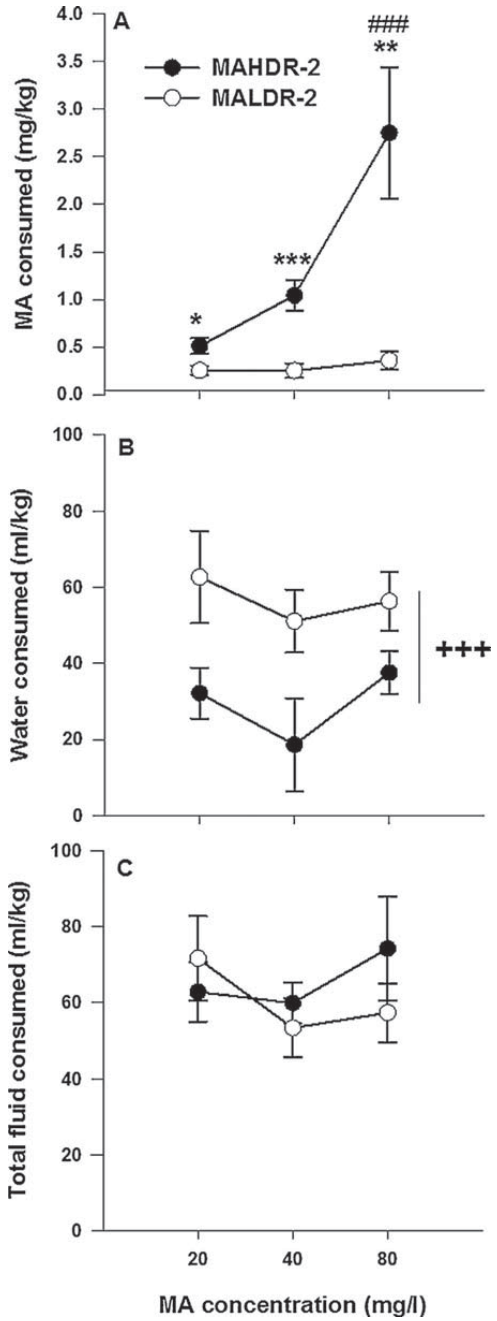


**Figure 4.1: Consumption of MA and water in MADR mice, when offered in a 4-h limited access two-bottle choice study.** Shown is (A) mean ± SEM mg/kg MA consumed (20, 40, 80 and mg/l), (B) mean ± SEM ml/kg water consumed, and (C) mean ± SEM ml/kg total fluid intake. Each data point is the group average for day 4 consumption for each MA concentration offered. *p<0.05, **p<0.01, ***p<0.001 for the difference between the MAHDR-2 and MALDR-2 line mice at each MA concentration. ###p<0.001 for the difference between the amount of MA consumed

at the 80, compared to 20 mg/l concentration within the MAHDR-2 line. +++p<0.001 for the main effect of selected line. N=17-18/line.

**Temporal pattern of licks from MA containing bottle**

Lick data were next considered in 30-min blocks of time for the final 4-h drinking sessions for each MA concentration to determine whether there were periods of heightened MA drinking behavior (Fig. 4.2). For the MA-containing tube (Fig. 4.2A, B, and C), there was a main effect of line ($F[1,30]=12.2$; $p<0.01$) and time bin ($F[7,210]=3.4$; $p<0.01$), but no effect of MA concentration or any significant interactions. Fig. 4.2D shows the total number of licks for each MA concentration. MAHDR-2 line mice took a greater number of licks from the MA-containing tube compared to MALDR-2 line mice, regardless of MA concentration ($F[1,30]=12.4$; $p<0.01$ for the main effect of line). For the water-containing tube, there were no statistically significant findings (Data not shown). Pearson's r correlations were calculated to examine the relationship between total licks and total volume for each MA concentration, and all were significant ($r=0.69$-$0.78$; $p<0.05$).

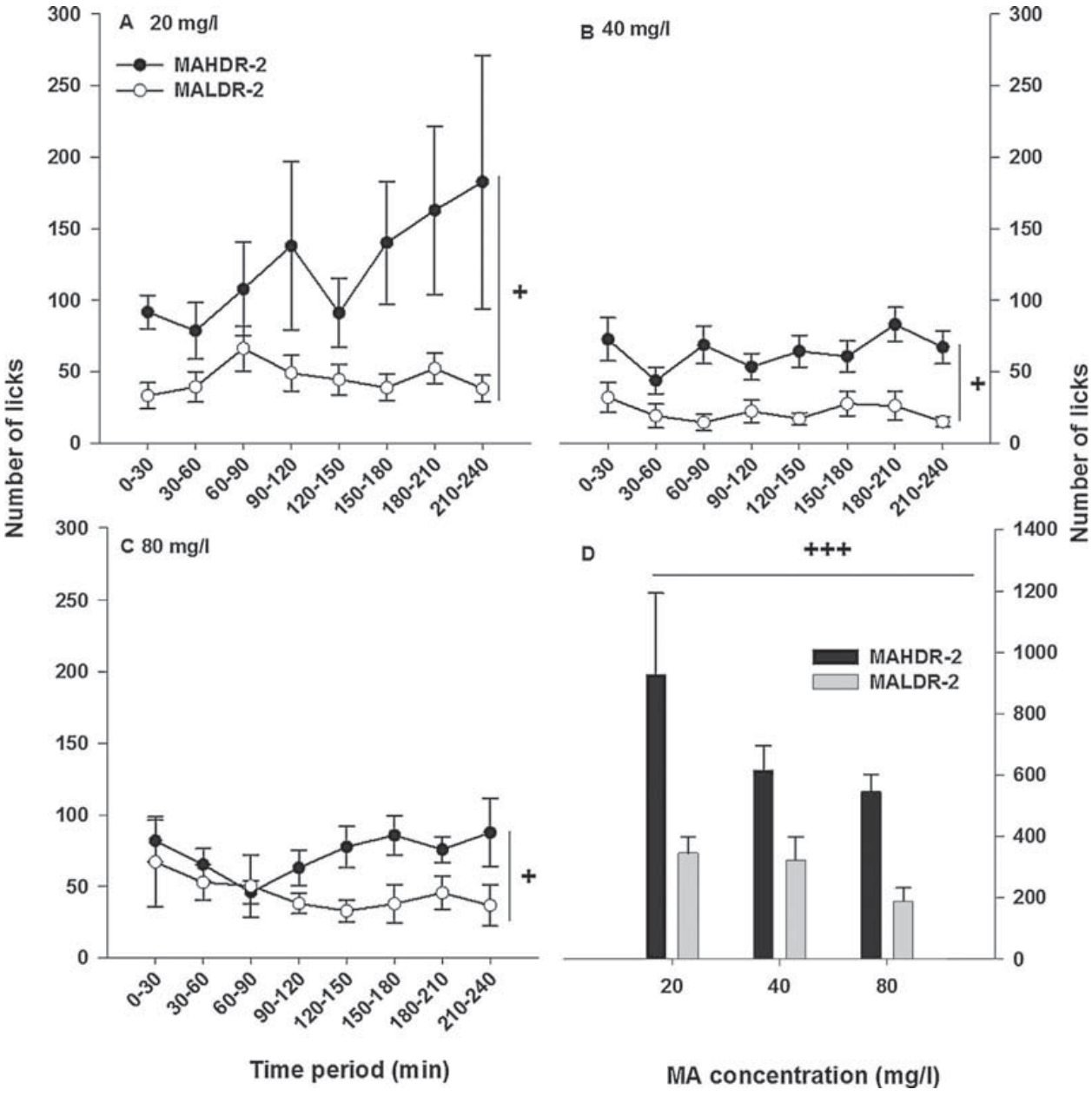


**Figure 4.2: Temporal pattern of licks taken from the MA-containing bottle.** Shown is mean ± SEM number of licks across time taken from the MA-containing bottle at increasing concentrations of MA; (a) 20 mg/l, (b) 40 mg/l, and (c) 80 mg/l. Data are shown in 30-min increments for day 4 of each MA concentration. (d) Mean ± SEM total number of licks taken

from the MA-containing bottle, accumulated for the entire 4-h session. +p<0.05, +++p<0.001 for the main effect of selected line. N=17-18/line.

**MA drinking pattern characteristics**

Fig. 4.3 shows bout parameters for MA intake during the final 4-h MA drinking sessions for each MA concentration. MAHDR-2 mice had significantly more MA bouts, compared to MALDR-2 mice ($F[1,30]=17.8$; $p<0.001$) and the number of bouts significantly decreased as the MA concentration increased, regardless of line ($F[2,60]= 3.4$; $p<0.05$; Fig. 4.3A). However, MALDR-2 line mice had a significantly greater interbout interval, compared to MAHDR-2 line mice ($F[1,30]=4.7$; $p<0.05$; Fig. 4.3E) and took significantly longer to complete their first drug bout ($F[1,30]=6.5$ $p<0.05$; Fig. 4.3C). Overall, the latency to first bout increased as the concentration of MA was increased ($F[2,60]=3.6$; $p<0.05$; Fig. 4.3F), regardless of line. Analysis of the same bout parameters for the water-containing tube did not identify any significant differences between the selected lines (data not shown).

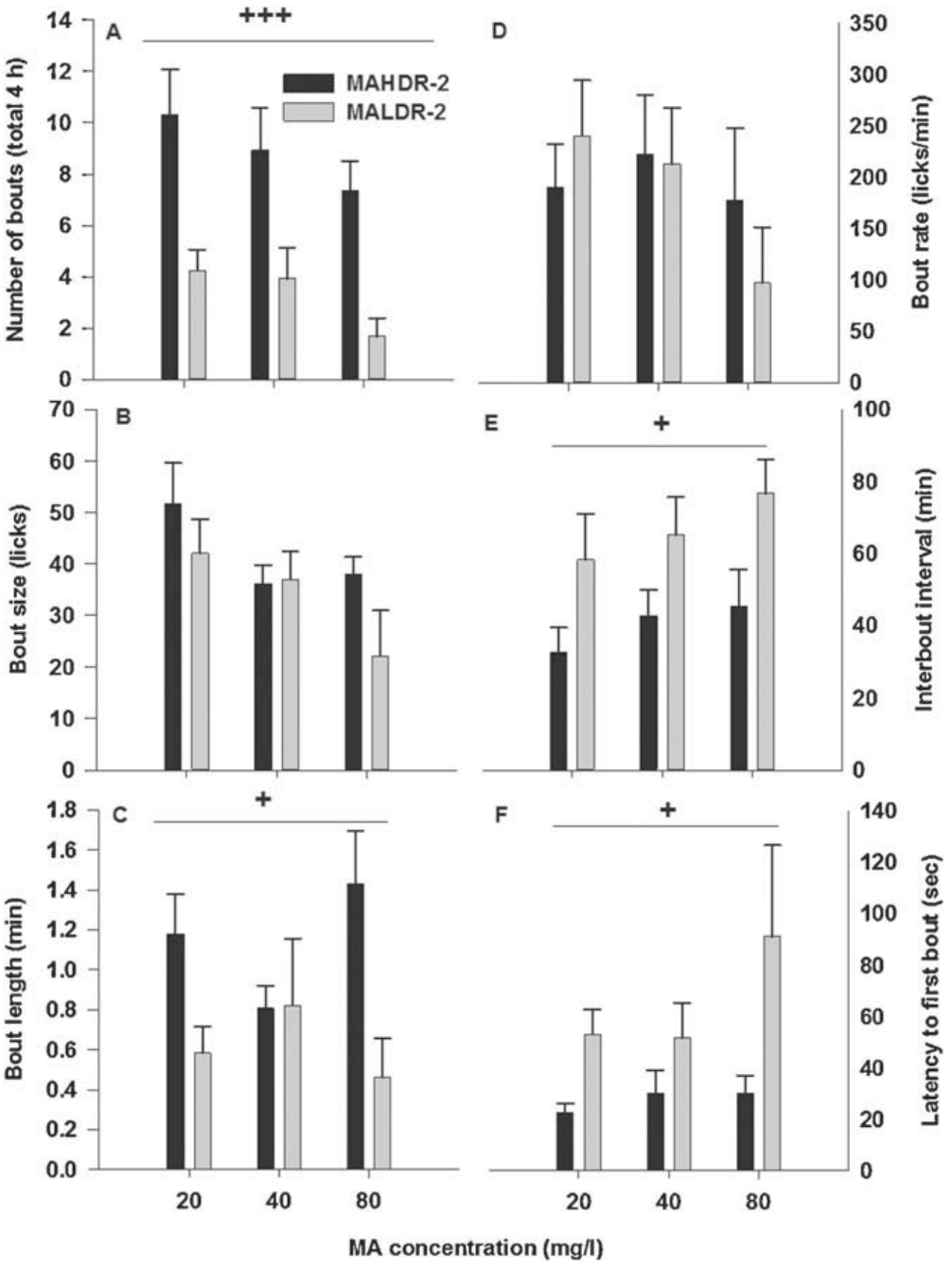


**Figure 4.3: MA drinking pattern characteristics.** Shown is mean ± SEM (a) number of bouts, (b) bout size, (c) length of bout, (d) bout rate, (e) interbout interval, and (f) latency to first bout for MAHDR-2 and MALDR-2 mice for day 4 of each MA concentration. +$p<0.05$, +++$p<0.001$ for the main effect of selected line. N=17-18/line.

Fig. 4.4 shows mg/kg MA consumed during the first four days of MA access, when MA was first offered (20 mg/l; days 3-6). This period was examined separately to detect changes in drinking patterns across initial access days, as previously examined in an oral operant MA self-administration procedure (Shabani S. *et al.*, 2012a). There was a significant line x day interaction ($F[3,90]=2.6$; $p<0.05$). Simple main effects analysis of the line difference on each day demonstrated that upon first access to MA, the two selected lines did not differ in MA intake, but a line difference emerged that was present on all subsequent days (all $p<0.05$). Simple main effects analysis of the difference across days within each line supported a significant increase in MA intake in MAHDR-2 mice ($p<0.05$ for the difference between day 6 and day 3), but no significant change across days in MALDR-2 mice, although there was a downward trend in MA intake. No differences in total volume consumed were identified during this 4-day period (data not shown).

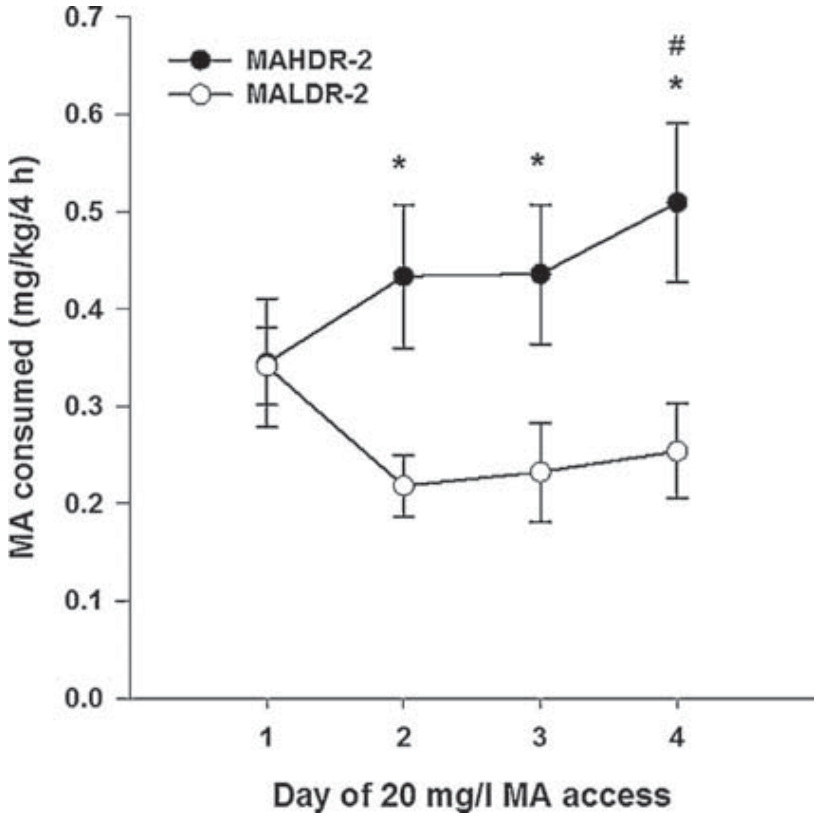


**Figure 4.4: MA intake across the first 4 days of MA access.** Shown is mean ± SEM mg/kg MA consumed during the first 4 days of MA (20 mg/l) access in MAHDR-2 and MALDR-2 mice. *p<0.05 for the difference between the MAHDR-2 and MALDR-2 line mice. #p<0.05 for the difference between day 4 and day 1 within the MAHDR-2 line. N=17-18/line.

**MA drinking patterns and characteristics across first 4 days of MA access**

Fig. 4.5 shows bout measures for the first 4 days of MA access. Compared to MALDR-2 mice, MAHDR-2 mice had a greater number of MA bouts ($F[1,30]=10.6$; $p<0.01$; Fig. 4.5A), longer bout length ($F[1,30]=10.4$; $p<0.01$; Fig. 4.5C), shorter interbout interval ($F[1,17]=10.0$; $p<0.01$; Fig. 4.5E) and shorter latency to first bout of MA drinking ($F[1,24]=6.2$; $p<0.05$; Fig. 4.5F). The interaction of line x day was not statistically significant for any of these measures, although Fig. 4.5 suggests changes in magnitude of the line difference over days. For MA bout size (Fig. 4.5B) and bout rate (Fig. 4.5D), there were significant line x day interactions ($F[3,90]=3.1$; $p<0.5$ and $F[3,90]=4.2$; $p<0.01$, respectively). MALDR-2 mice had a larger bout size, compared to MAHDR-2 mice, on day 1 of 20 mg/l MA access ($p<0.05$), but not on subsequent days of access to this concentration of MA. Additionally, on days 1 ($p<0.001$) and 2 ($p<0.05$) of 20 mg/l MA access, the MALDR-2 line had a greater bout rate, compared to the MAHDR-2 line, with this line difference disappearing by the third day of MA access. For water during the same time period, there were no differences in number of bouts, bout size, interbout interval, or latency to first bout (data not shown). However, MALDR-2 mice did have shorter length water bouts ($F[1,30]=7.4$; $p<0.05$ for the main effect of line; MALDR-2: 0.9±0.1 and 1.4±0.1 sec for MALDR-2 and MAHDR-2, respectively) and a quicker bout rate [$F(1,30)=38.6$; $p<0.001$ for the main effect of line; MALDR-2: 281.8±18.6 and 127.8±16.3 licks/min for MALDR-2 and MAHDR-2, respectively).

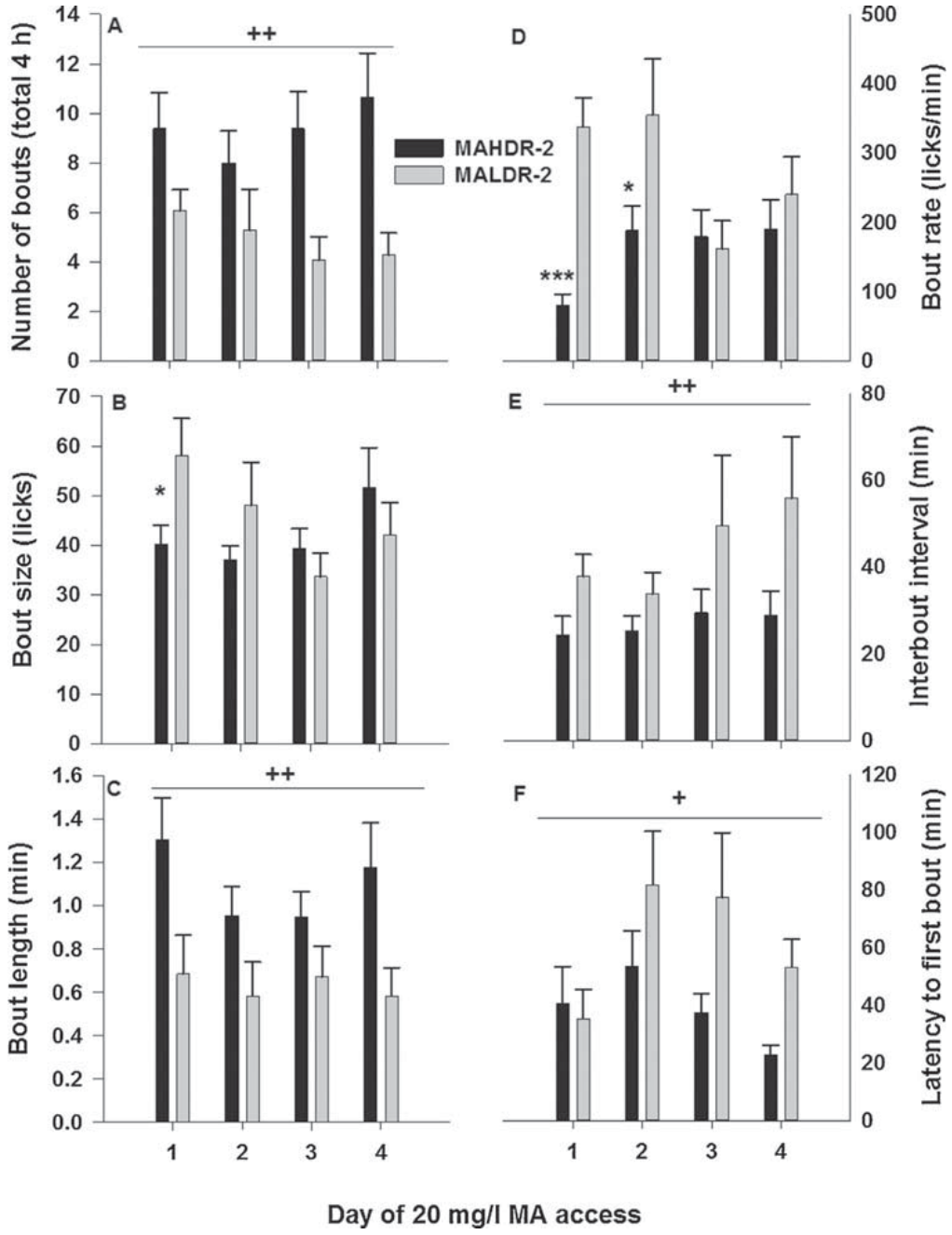


**Figure 4.5: MA drinking pattern characteristics across the first 4 days of MA access.**

Shown are mean ± SEM (a) number of bouts, (b) bout size, (c) length of bout, (d) bout rate, (e) interbout interval, and (f) latency to first bout for MAHDR-2 and MALDR-2 mice during the first 4 days of MA (20 mg/l) access. *$p<0.05$, ***$p<0.001$ for the difference between the MAHDR-2 and MALDR-2 line mice. +$p<0.05$, ++$p<0.01$ for the main effect of selected line. N=17-18/line.

**MA consumption and corresponding MA blood levels**

Fig. 4.6 shows MA intake and corresponding blood MA level data for 3 groups of mice that each had 3 blood samples obtained at particular times. For group 1 mice, blood was taken after the first day of MA intake (day 3), because it was predicted that the MADR-2 line mice would consume similar amounts of MA on this day and would also have similar blood MA levels. This group was also sampled on the final day that the lowest concentration was offered and on the final day of the study, times when MA intake was predicted to be higher in the MAHDR-2 line than on the first day and lower in the MALDR-2 line. Data for this group were analyzed separately from groups 2 and 3, because mice in those groups they were not sampled at the same times. For MA intake (Fig. 4.6A), there was a significant main effect of line ($F[1,11]=4.9$; $p<0.05$) and of day ($F[2,22]=8.1$, $p<0.001$), but the line x day interaction was not statistically significant ($p=0.15$). Thus, patterns of increasing intake in the two selected lines as the MA concentration was increased were not significantly different; however, greater MA intake in MAHDR-2 mice confirmed the trait for which they were bred. For blood MA levels in group 1 animals (Fig. 4.6B), there was a significant line x day interaction [$F(2,22)=8.6$; $p<0.01$]. For the simple main effect of line at each of the sampling days, there was no significant difference between the lines on day 3, but the MAHDR-2 line mice had significantly greater blood MA concentrations than MALDR-2 line mice on days 6 and 14 ($p<0.05$ and $p<0.01$, respectively). With regard to differences across day within line, there was a significant simple main effect of day within the MAHDR-2 line; MAHDR-2 mice had significantly higher blood MA levels on day 14 compared to day 3 ($p<0.001$). There was no significant simple main effect of day within the MALDR-2 line, indicating that blood MA levels were comparable across days in these mice.

Group 2 and 3 mice were sampled on the same 3 days at either 2 h or 4 h into the limited access session (Fig. 4.6C and D). Data for these groups were statistically compared. For MA consumption, there was a significant main effect of line [$F(1,25)=15.5$; $p<0.001$] and of day [$F(2,50)=3.5$; $p<0.05$], but no significant line x day interaction. MAHDR-2 mice consumed

significantly more MA than MALDR-2 mice, regardless of concentration, but more MA was consumed as the concentration of MA was increased (Fig. 4.6C). There was also a significant main effect of sampling time [$F(1,25)= 7.7$; $p<0.01$]; more MA was consumed at the 4 h compared to the 2 h time point. For the corresponding blood MA level data (Fig. 4.6D), there was no significant difference in amount of MA in samples obtained at 2 vs 4 h (group 2 compared to group 3). However, there was a significant line x day interaction [$F(2,54)= 3.5$; $p<0.05$]. The simple main effect of line at each day was significant; MAHDR-2 mice had significantly higher blood MA levels on each day ($p<0.05$, 0.001, and 0.001, respectively). In addition, the simple main effect of day within the MAHDR-2, but not MALDR-2 line, was significant; MAHDR-2 mice had significantly higher blood MA levels after consuming the 40 and 80 mg/l MA concentrations, compared to the 20 mg/l MA concentration ($p<0.05$ for both comparisons). A significant correlation (Pearson's r; $r=0.58$; $p<0.05$) was found between established MA intake (days 6, 10 and 14) and blood MA levels obtained after consumption of each MA concentration. The correlation was comparable whether calculated using all data from days 6, 10 and 14, or for each MA concentration independently or for the 2-h vs. 4-h drinking period groups.

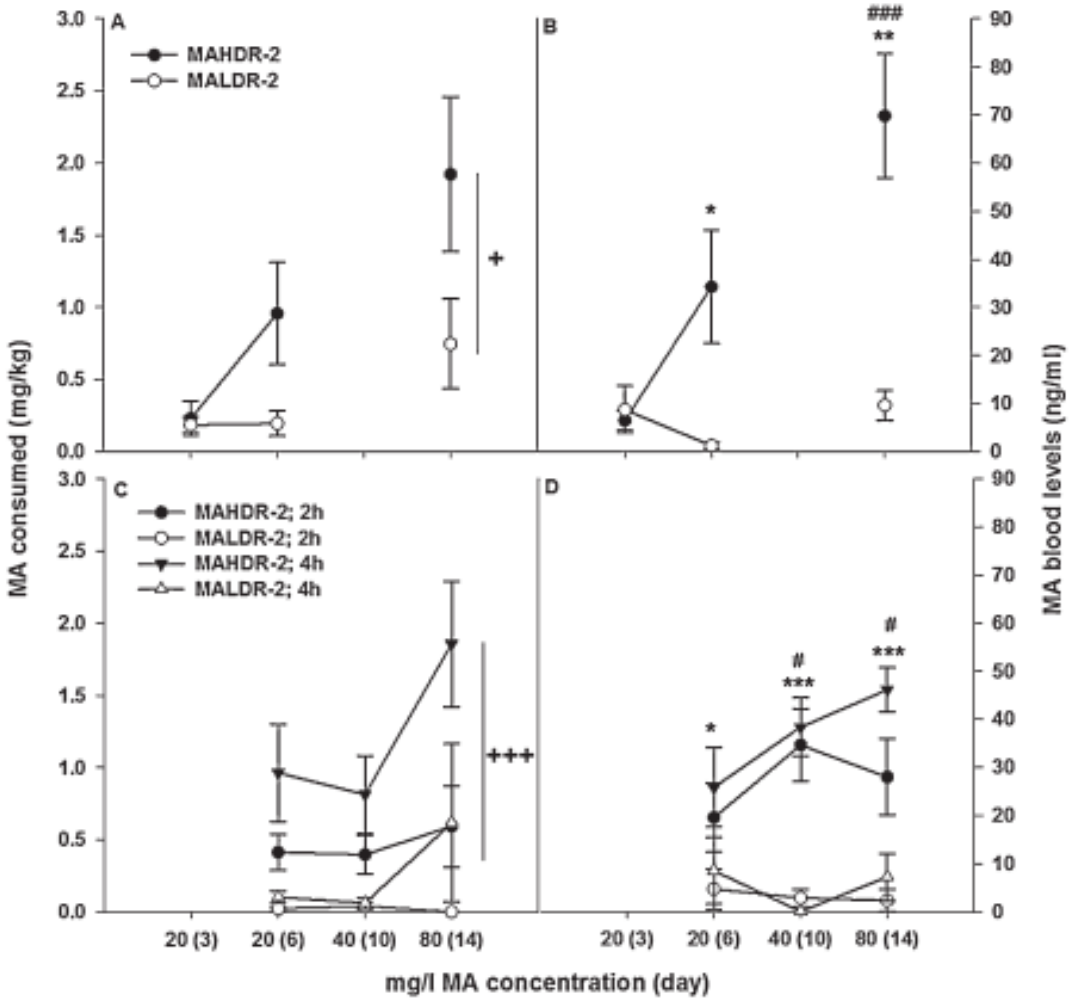


**Figure 4.6: MA consumption and corresponding blood MA levels in MAHDR-2 and MALDR-2 mice.** All data shown are means ± SEM. Group 1 data from the relevant study are shown in (a) for mg/kg MA consumed and (b) blood MA levels (ng/ml) on the first (day 3) and fourth (day 6) day of MA access (20 mg/l) and the fourth (day 14) day of MA at the highest concentration offered (80 mg/l). Group 2 and 3 data are shown in (c) for mg/kg MA and (d) blood MA levels (ng/ml) on the fourth day that each MA concentration was offered (day 6, 10 and 14), at either 2 or 4 h into the 4-h limited access procedure. *$p<0.05$,**$p<0.01$, ***$p<0.001$ for the MADR line difference. #$p<0.05$, ###$p<0.001$ for within-group difference between MAHDR-2 mice at the 40 and 80 mg/l MA concentrations and the 20 mg/l MA concentration. +++$p<0.01$ for the main effect of line. N=6-9/line/group.

## DISCUSSION

These data demonstrate that selective breeding for MA intake has resulted in differences in MA drinking characteristics between the high and low MADR mouse lines, some of which appear during the first drinking session. The MAHDR-2 line consumed more MA at each concentration of MA offered, compared to the MALDR-2 line, while the MALDR-2 line consumed more water compared to the MAHDR-2 line, resulting in comparable levels of total fluid consumed. MAHDR-2 line mice had a greater number of MA drinking bouts, longer bout duration, shorter interbout interval, shorter latency to first MA drinking bout and greater number of licks from the MA-containing bottle, compared to the MALDR-2 line. On the first day of MA access, the MADR-2 lines did not differ in MA consumption, but with subsequent access, the MAHDR-2 line escalated their MA intake, while the MALDR-2 line showed a pattern of decreasing MA intake, although this decrease was not statistically significantly. Although total intake did not differ on the first day, analysis of bout parameters during the initial 4-d MA access period showed that the MAHDR-2 line, regardless of day, had a greater number of and longer MA bouts, shorter interbout interval, and shorter latency to first MA bout, compared to the MALDR-2 line. In a separate study, MA consumption and blood levels were similar for the 2 lines on the first day of MA access, but the MAHDR-2 line mice had higher MA intake and significantly increased blood MA levels as the MA concentration in the drinking solution was increased. Results from the lickometer study, identified differences in intake parameters prior to MA intake differences. Significant differences between the lines across days of the initial access period were found only for bout size and rate.

A previous study demonstrated that the MADR-2 lines do not differ in rate of MA clearance from blood following a bolus 2 mg/kg MA injection (Shabani S. *et al.*, 2012b). Peak MA levels (~400 ng/ml) were observed 15 minutes following MA administration, with peak level somewhat higher in MAHDR-2 than MALDR-2 mice and almost complete clearance within 2 h later. In the current study, when MADR-2 mice were allowed to voluntarily consume MA during

4-h periods, MA blood levels for individual animals were between 0 and100 ng/ml. These levels were likely impacted by drinking pattern, concentration of the MA solution, and when during the session the samples were obtained.

A previous study examined locomotor activity following a 1 h operant MA self-administration study in MADR-2 mice (Shabani S. *et al*., 2012b). On average, MAHDR-2 line mice consumed 0.4-0.7 mg/kg MA and MALDR-2 line mice consumed 0.1-0.2 mg/kg MA. These consumption values corresponded with level of activity, when locomotor activity data were collected on several days, within 15 min after the conclusion of the drinking sessions, suggesting that this level of MA intake was behaviorally relevant. In the current study, the MADR-2 lines consumed greater amounts of MA (up to 2 mg/kg on average for one group of MAHDR-2 mice and 0.75 mg/kg in one group of MALDR-2 mice), consistent with longer drinking sessions and the higher 80 mg/l MA concentration offered here that was not used in the previous study. In human subjects, a low to moderate dose of MA (5-30 mg or 0.06-0.4 mg/kg based in a 75 kg human) is known to produce euphoric subjective effects, whereas doses above 50 mg (or above 0.7 mg/kg in a 75 kg human) have been shown to induce euphoria followed by psychosis (Bell D. S., 1973; Cruickshank C. C. & Dyer K. R., 2009). We did not see large differences in blood MA levels in mice sampled at 2 vs 4 h, and the correlation between intake and MA levels was comparable for these groups. This may indicate that the mice consume MA in a pattern that titrates blood MA to a certain level and subjective effect.

It is of particular importance that the selected lines did not differ in MA drinking on their first day of MA access. In a previous examination of operant oral self-administration of MA, a similar outcome was obtained during a 1-h long trial (Shabani S. *et al.*, 2012b). However, in that study a saccharin fading procedure was used, so that MA was initially offered in a saccharin-sweetened solution. The current findings are of further interest, because the solutions offered were not sweetened to incentivize consumption, yet an almost identical outcome was obtained. In addition, the blood MA data supported a lack of difference between the MADR-2 lines in MA

level after this first MA drinking session. Using a two-bottle choice procedure, we have found no differences in taste preference for bitter or sweet solutions between the MADR lines (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). The current data suggest that the MALDR-2 line does not initially avoid consuming the MA-containing solution due to its bitter taste qualities. Rather, it appears that they must first experience the pharmacological effects of MA before choosing to avoid consumption. Findings that support extreme sensitivity of MALDR mice to aversive effects of MA, using conditioned place preference, conditioned place aversion and conditioned taste aversion (CTA) procedures (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011; Shabani S. *et al.*, 2012a; Shabani S. *et al.*, 2012b), suggest the possibility that aversive effects of MA were experienced by the MALDR-2 mice during the first MA drinking access session.

It has been demonstrated that ethanol, under some conditions and particularly in low preferring strains, can produce conditioned aversion during acquisition of ethanol consumption (Cannon D. S. & Carrell L. E., 1987b, 1987a). Cannon and Carrell (1994) examined several rat strains with high and low ethanol preference and obtained data suggesting that initial preference and pattern of initial consumption were related to subsequent patterns of intake. Strains that ultimately showed low ethanol preference tended to have consumed larger amounts of ethanol during their first access session, while those that ultimately exhibited higher preference had low to moderate initial ethanol intake, and continued to consume ethanol at about the same or at a higher level. Although the MADR lines initially consumed equivalent amounts of MA, due to the much higher sensitivity of the MALDR line to the aversive effects of MA, this amount could have induced taste aversion, resulting in reduced intake thereafter. Profound MA-induced CTA is seen at 1 mg/kg MA in MALDR mice, the lowest dose we have tested, and no CTA is seen in MAHDR mice even at a dose of 4 mg/kg). This difference between the MADR lines in sensitivity to MA-induced CTA appears to be specific to MA, as the lines show similar patterns of CTA development for cocaine (Gubner N. R. *et al.*, 2013) and ethanol (Phillips, unpublished). It is of interest to examine aversive effects in the MALDR mice at doses as low as those they

voluntarily consume (0.3 – 0.5 mg/kg). The lines do not differ significantly in rate of MA clearance (Shabani S. *et al.*, 2012b). Further, the MADR lines do not differ in their oral preferences for salty (KCl; NaCl), bitter (qunine), or sweet (saccharin) drinking solutions (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). These findings for MA support the hypothesis that initial exposure to a drug is a particularly salient experience that influences future drug taking patterns.

To our knowledge, the microstructure of oral MA intake in an animal model has not been previously documented. However, such data have been generated for ethanol intake in some genotypes of rat and mouse (Samson H. H. *et al.*, 1988; Samson H. H., 2000; Ford M. M. *et al.*, 2005; Rhodes J. S. *et al.*, 2007; Ford M. M. *et al.*, 2009; Barkley-Levenson A. M. & Crabbe J. C., 2012). Those studies have supported a positive correlation between larger bout size and higher ethanol intake (g/kg). In addition, a similar finding has been demonstrated in a non-human primate model, in which classification of subjects as “sippers” or “gulpers” was predictive of later ethanol intake patterns (Grant K. A. *et al.*, 2008). We observed only a transient difference in MA bout size between our high and low MA consuming lines in the current study (day 1 only). Instead, a larger number of bouts, greater bout duration, and shorter interbout interval appeared to play a significant role in the greater MA consumption of MAHDR-2, compared to MALDR-2, mice. It has been suggested for ethanol that length of interbout interval and bout frequency indicate magnitude of “craving” (Samson H. H. *et al.*, 1988). Whether this is the case for MA will require additional study, perhaps using a model of extinguished use followed by relapse. Such studies in our genetic animal model of higher MA intake may provide insights into patterns of human MA use

We identified significant correlations between volume consumed and number of licks (r=0.69-0.78). Data were collected for all 4 consecutive days that each MA concentration was offered. However, stronger correlations were found when day 4 values alone were used, which led, in part, to our decision to focus on day 4 data for each MA concentration. Others have

reported somewhat larger volume-lick correlations for ethanol drinking (e.g., r=0.87-0.97). However, in those studies, the rodents resided in isolate housing in the lickometer chambers, 24 h per day throughout the study, and were given a 7-d acclimation period in the lickometer chambers before beginning the experiment (Ford M. M. *et al.*, 2005; Rhodes J. S. *et al.*, 2007; Ford M. M. *et al.*, 2009). We wished to avoid isolate housing in the current limited access study, and placed the mice into the lickometer chambers for each daily drinking session and then returned them to home cages with same-sex littermates. Our selection protocol for MA consumption isolate houses mice for the 10-d selection procedure. The current data show that the MADR lines consume different amounts of MA when not chronically isolate housed and during a limited access procedure. This is consistent with other limited MA access data for two-bottle choice under isolate housing conditions (Eastwood E. C. & Phillips T. J., 2014) and for operant MA self-administration without chronic isolate housing (Shabani S. *et al.*, 2012a), and indicates that the difference in MA intake between the lines is consistent across multiple procedures.

## CONCLUSIONS

Our data illustrate that genetic susceptibility to MA consumption corresponds with a larger number of MA drinking bouts, a greater bout duration, a shorter latency to first MA bout, a shorter interbout interval, and higher blood MA levels. These MA drinking characteristics could be associated with greater genetic risk for MA dependence. A comparison of binge (MA use up to 22 times/day for 4-6 days) and non-binge patterned MA users that used an equivalent amount of MA over a 30-d period, demonstrated that binge patterned users were more likely to suffer from health, social, and behavioral consequences compared to non-binge patterned MA users (Semple S. J. *et al.*, 2003; Sommers I. *et al.*, 2006). In the current study, we examined consumption of a more concentrated solution of MA (80 mg/l) than used previously (up to 40 mg/l). The MAHDR-2 line showed a marked increase in MA intake and blood MA levels, when MA was offered as an 80 mg/l solution, then when offered as a 20 or 40 mg/l solution. In part,

this may be because a higher dose can be attained by consuming similar volume. However, because there was always a water choice, the mice could have chosen to reduce their intake if they had found the dose to be aversive. In this article, we show day 4 data for each concentration, indicating that the mice did not avoid consuming a high dose of MA on their final day of access. In an 18-h period (6 h during the light and 12 h during the dark), MAHDR mice consume ~6 mg/kg MA from a 40 mg/l solution. Here they consumed a dose of ~3 mg/kg in only 4 h (during the dark). Our future plans include further development of a binge-like model of MA intake over a more chronic period to better model human MA dependence.

# CHAPTER 5:

## Opioid Treatment to Reduce Oral Methamphetamine Intake and Verification of a Methamphetamine Intake QTL

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*[This chapter contains unpublished data.]*

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**Abstract**

A major effect quantitative trait locus (QTL) for methamphetamine (MA) consumption has been mapped to proximal chromosome (Chr) 10, based on genetic and phenotypic variation in mice selectively bred for high (MAHDR) or low (MALDR) levels of MA drinking. The partial MOP-r agonist drug, buprenorphine, reduced MA intake in the MAHDR line in previous research. Here, we investigated the impact of the full MOP-r agonists, morphine and fentanyl, on MA intake, and of morphine on saccharin intake, in 2-bottle choice drinking procedures. MOP-r density and affinity were measured in several brain regions from the MA drinking lines of mice and their progenitor strains, using [$^3$H]DAMGO binding. Finally, MA intake was measured in two congenic strains of mice with different length segments of C57BL/6J (B6) DNA on a DBA/2J (D2) inbred strain background on Chr 10 (Chr 10 D2.B6 0-7.72 and Chr 10 D2.B6 0-20.4 Mb), both containing *Oprm1*. At some doses, morphine and fentanyl reduced MA intake in the MAHDR line, but reductions in saccharin intake and total volume consumed were also found. Consistent with greater *Oprm1* expression in medial prefrontal cortex (mPFC) tissue from MALDR, compared to MAHDR, mice, MALDR mice also had greater MOP-r density in the mPFC. The Chr 10 D2.B6 0-20.4 Mb congenic, but not 0-7.72 Mb congenic differed from the D2 background strain for MA intake. These data suggest that full opioid receptor agonists induce reduce MA drinking, but may do so by inducing competing behavioral effects. Greater MA intake is associated with a lower density of MOP-rs in the mPFC, but *Oprm1* was not confirmed as a quantitative trait gene influencing MA intake.

## 1. INTRODUCTION

Use of methamphetamine (MA) has debilitating consequences and may be genetically influenced. There are currently no Federal Drug Administration approved pharmacological interventions to treat MA dependence. We have used selective breeding to produce lines of mice that exhibit high (MAHDR) or low (MALDR) MA drinking levels (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2012b). In 3 independent sets of replicate MA drinking (MADR) lines (MADR-1, MADR-2, and MADR-3), the MAHDR line consumed ~6 mg/kg of MA during an 18-h period, when a 40 mg/l concentration of MA was offered, while the MALDR line consumed ~0.5 mg/kg of MA (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011) Phillips et al., 2015 unpublished data). Genetic mapping to identify regions of chromosomes (Chr) containing genes that are polymorphic between the MADR lines detected a major effect quantitative trait locus (QTL) on proximal mouse Chr 10 that explains greater than 50% of the genetic variance in MA intake between the MADR mouse lines (Belknap J. K. *et al.*, 2013). A global gene expression analysis in medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and ventral midbrain (Vmb) tissue from drug-naïve MADR mice identified many genes that were DE. One such gene is the μ-opioid receptor (MOP-r) gene, *Oprm1,* which resides within the confidence interval of the Chr 10 QTL for MA intake, with the MALDR having greater expression in the mPFC, but not NAc or Vmb, compared to MAHDR mice (Belknap J. K. *et al.*, 2013). In addition to the gene expression data, pharmacological treatment with MOP-r agonist and antagonist drugs has been shown to attenuate MA intake and other MA-related traits in humans, non-human primates, and rodents (Jones D. N. & Holtzman S. G., 1994; Jayaram-Lindstrom N. *et al.*, 2004; Jayaram-Lindstrom N. *et al.*, 2005; Ide S. *et al.*, 2006; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b; Dlugos A. M. *et al.*, 2011; Eastwood E. C. & Phillips T. J., 2014). *OPRM1* has also been associated with MA-induced psychosis and dependence. Collectively, these data

supported our interest in *Oprm1* as a candidate gene within the QTL interval (Ide S. *et al.*, 2004a; Ide S. *et al.*, 2006).

Our previous studies identified MOP-r-related MADR line differences, with the MALDR line having greater MOP-r agonist-stimulated locomotor activity and greater avidity for the MOP-r agonist drug, morphine (MOR), compared to the MAHDR line (Eastwood E. C. & Phillips T. J., 2012). Additionally, we found that lower doses of buprenorphine (BUP), a partial MOP-r agonist reduced the amount of MA consumed by MAHDR mice, in the absence of effects on total volume of fluid consumed (Eastwood & Phillips, 2014). In that study, acquisition, rather than effects on established drinking, was evaluated to explore the role of MOP-r in the development of MA intake. An inverted U-shaped dose-response curve was found for the effect of BUP and we hypothesized that at lower doses, agonist actions at the MOP-r reduced MA intake, whereas at higher doses, antagonist actions at the MOP-r known to occur with BUP increased MA intake.

To determine if full MOP-r agonist drugs, devoid of antagonist activity, would linearly reduce MA intake during acquisition, the effects of MOR and fentanyl (FENT) were examined. MOR was selected because it is a selective MOP-r agonist, but also because we have previously examined MOR intake, nociception, and acute locomotor activation in the MADR mice (Eastwood & Phillips 2012, 2014). FENT was used because it is also a selective MOP-r agonist, but has greater pharmacological specificity for the MOP-r than MOR (Kalvass J. C. *et al.*, 2007). The effect of MOR pretreatment was examined in the same 6-h limited access drinking procedure used to examine the effects of BUP (Eastwood & Phillips, 2014). The effect of MOR on saccharin intake was also assessed using the same procedure, to address whether MOR effects were specific to MA intake. Because FENT has shorter pharmacological actions, a lickometer apparatus and a 4-h session were implemented to better capture its effects. Drinking patterns and microstructural features of drinking were examined in the FENT study, as previously examined for MA drinking in the MADR lines (Eastwood E. C. *et al.*, 2014).

Because differences in MOP-r sensitivity and *Oprm1* expression between the MADR lines have been detected, differences in MOP-r density and affinity were assessed between the MAHDR and MALDR lines, using [$^{3}$H]DAMGO, a radiolabeled MOP-r agonist. MOP-r density and affinity were also assessed in the progenitor inbred mouse strains that served as founders of the selected lines, the C57BL6/J (B6) and DBA2/J (D2) strains, to determine if the selected lines resemble their parental strains on this measure. The mPFC, NAc, and Vmb regions were examined, to align with the previous gene expression analysis in MADR mice (Belknap J. K. *et al.*, 2013).

Finally, MA intake was examined in congenic mice for the purpose of confirming the Chr 10 QTL on an isogenic background, and possibly reducing the size of the QTL interval. Two congenic strains of mice were available that were derived from the B6 and D2 progenitor strains. The congenics had differing length Chr 10 segments from the B6 strain (donor) introgressed onto the D2 strain (recipient) background. The D2 strain consumes more MA, compared to the B6 strain (Eastwood E. C. & Phillips T. J., 2014) and B6 alleles in the Chr 10 QTL are associated with reduced MA intake (Belknap J. K. *et al.*, 2013). Thus, if one or more genes in the introgressed B6 region has a role in MA intake, then the congenic strain should have reduced MA intake compared to the background D2 strain. A reduction in MA intake of a similar magnitude in both congenic strains would indicate that the relevant gene(s) can be found within the boundaries of the smaller donated B6 segment. Reduced MA intake in only the larger segment congenic strain, would verify the presence of one or more influential genes within the boundaries of the introgressed segment and also eliminate the overlapping segment of the 2 congenic strains from further consideration. Finally, no significant reduction in MA intake in either strain would indicate that the QTL is not contained within the region of the introgressed segment or that an epistatic interaction (interaction with one or more other genes in the genetic background) plays a role in its effect.

**METHODS**

**Animals and Husbandry**

The MADR mouse lines were created using mass selection in a short-term selective breeding project. Detailed methods have been published for the consecutive production of the first and second sets of replicate mouse lines. For each selection, B6 and D2 mice were crossed to create an F2 population and 120 individuals were tested in an 18-h, two-bottle choice MA vs water drinking procedure, in which 20 mg/l then 40 mg/l MA in water was each offered for 4 consecutive days. The 13 pairs of highest and 13 pairs of lowest MA consuming individuals were selected as breeders and used to establish the MAHDR and MALDR mouse lines, respectively. The first litter offspring were tested for MA drinking and this process was continued for five total selection generations. This selection procedure has produced similar differences in MA drinking in three sets of replicate MADR lines that were created at approximately 2-year intervals (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011) Phillips et al., 2015, unpublished data).

MADR mice used in the current studies were second or later litter MA-naïve offspring of the fifth selection generation of replicate 2 MADR mouse lines. They were weaned at age 21-23 days and then group-housed with 2-5 same sex littermates. When necessary to avoid singly housing mice, singleton offspring were group-housed with other same sex non-littermates of the same approximate age (±5) and line. Mice were housed in shoebox cages (28.5 x 17.5 x 12 cm) with Bed-O-Cob™ bedding (The Anderson Inc., Maumee, OH) and wire cage tops, and maintained at 21±1°C.

The congenic strain mice that served as breeders to produce the mice used in this study were received from the laboratory of Dr. Wade Berrettini (University of Pennsylvania) and have been previously described (Berrettini W. H. *et al.*, 1994b; Ferraro T. N. *et al.*, 2005). Briefly, these strains were derived from B6 and D2 mice that were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice from the F1 intercross of B6 x D2 were backcrossed to the D2 strain twice to form an N2 population. N2 generation mice were genotyped and subsequent

breeders for the next round of backcrossing were selected based on heterozygosity at particular polymorphic microsatellite DNA markers on Chr 10. This process was repeated until genotyping confirmed an introgressed B6 segment of particular length on the D2 background (D2.B6 mice). Breeder mice received in our laboratory were male and female mice that were heterozygous for the Chr 10 D2.B6 0 - 7.72 Mb segment mice and one male that was heterozygous for the Chr 10 D2.B6 0 - 20.4 Mb segment. The single male was mated with female D2 mice to obtain heterozygous male and female offspring. To produce mice for studies and maintain the congenic strains, heterozygote x heterozygote matings were performed to produce three possible genotypes; homozygous or heterozygous for the B6 introgressed region and pure D2. DNA was extracted from tail snips and congenic strain mice were genotyped using polymorphic microsatellite markers on Chr 10 for the B6 and D2 strains. The presence of a particular polymorphism was determined using PCR amplification followed by electrophoresis on a 4% agarose gel stained with ethidium bromide. The length of the donor segment for each congenic strain was determined by identifying where the transition from B6 markers to D2 markers occurred. Because there is some distance between the 2 markers at this transition point, a small interval is of unknown genotype. The congenic strains were considered to be genetically identical, except for a 12.86 Mb non-overlapping region of B6 DNA and transition region from 7.58 to 20.4 Mb on Chr 10. The schematic diagram for Chr 10 in the 2 congenic strains is shown in Fig. 5.1.

For all drinking studies, mice were placed on a reverse 12:12 h light:dark cycle (lights off at 0700 h and on at 1900 h) at least 2 weeks before the study began, with unlimited access to water and standard rodent diet (Purina 5001TM, Animal Specialties Inc., Hubbard, OR) at all times. The reverse light:dark cycle was implemented so that drinking behavior could be monitored during the dark phase, when mice are most active and engage in most of their consummatory behavior. All procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were

approved by the Institutional Animal Care and Use Committee of the VA Portland Health Care System. Numbers of animals tested of each sex and their ages are given with details for each experiment.

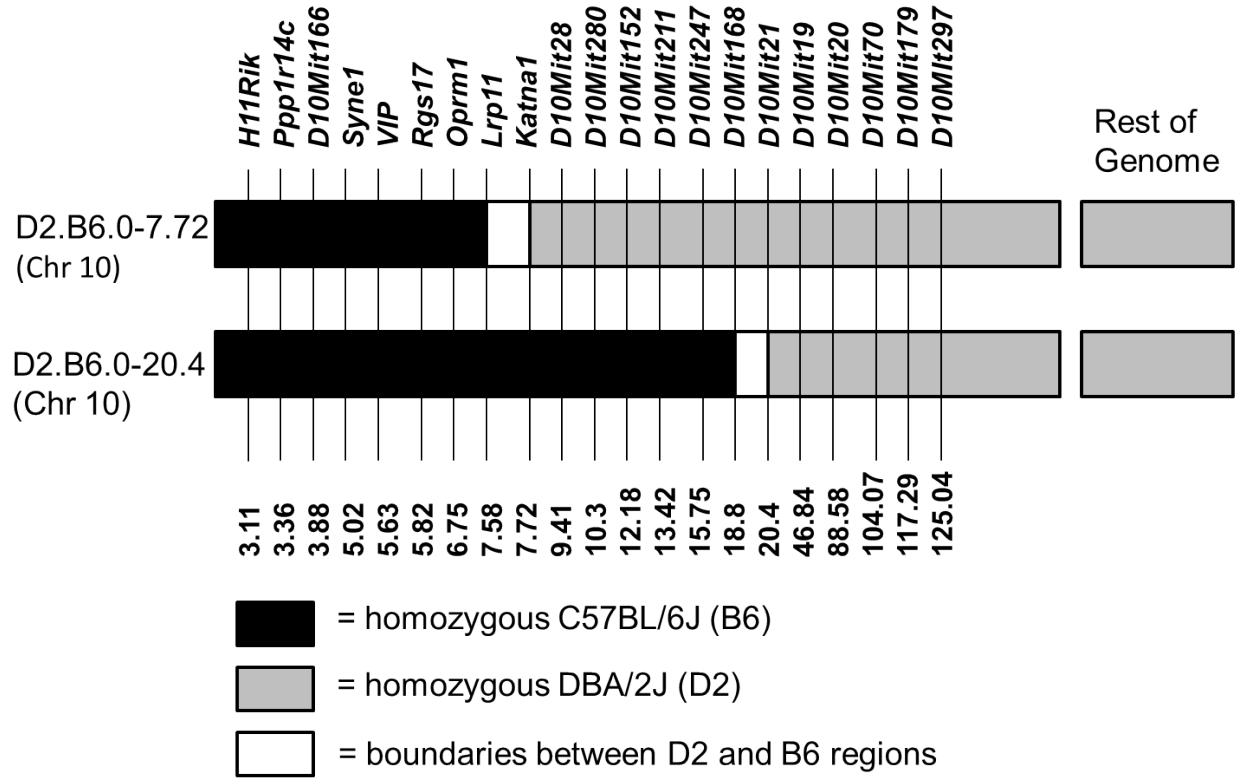


**Figure 5.1. Diagram representing the genotype of the Chr 10 D2.B6 0-7.72 (top) and D2.B6 0-20.4 (bottom) congenic strains.** Each congenic strain had a DBA2/J (D2) background genotype on all chromosomes, with a C57BL6/J (B6) segment only on mouse Chr 10. Grey regions reflect D2 strain genome and black regions reflect B6 strain genome. The white segments represent boundaries between confirmed B6 and D2 genome and are of unknown genotype. Above the diagram are the names of DNA microsatellite markers used to identify the length of the introgressed segment, and below are the corresponding genetic map positions in Megabases (Mb).

**Drugs**

(+) MA hydrochloride (HCl), saccharin sodium salt, [D-Ala$^2$, NMe-Phe$^4$, Gly-ol$^5$]-enkephalin [DAMGO], and FENT citrate were purchased from Sigma (St. Louis, MO). Morphine sulfate and [3H]DAMGO was obtained from the NIDA drug supply program (Bethesda, MD). For drinking solutions, MA, and saccharin were dissolved in tap water. For injections, MOR and FENT were dissolved in saline (0.9% NaCl, Baxter Healthcare Corporation, Deerfield, IL). All injections were given intraperitoneally (IP) at a volume of 10 ml/kg.

**Effect of MOR on MA two-bottle choice drinking**

A 12-day procedure, identical to that used to examine the effect of BUP on the acquisition of MA drinking (see Eastwood and Phillips, 2012), was utilized. Mice were 71-97 days old and data were collected in 4 cohorts (final N=7-9/sex/line/dose; n=2-3/sex/line/dose per cohort). A limited-access drinking procedure was used to capture the effects of the drug pretreatments in a time frame that corresponded with drug half-life. Mice were isolate housed on day 1 and offered two, 25-ml tubes containing tap water for 2 days to acclimate to the drinking apparatus. On days 3 and 4, mice received saline injections immediately before dark phase onset to familiarize them with handling and injection. On day 5, saline or MOR (5, 10, or 15 mg/kg) was administered immediately before dark phase onset, and water and 20 mg/l MA were offered for a 6-h period. The doses of MOR were based on previous experiments measuring effects of MOR on locomotor activity in MADR-2 mice (Eastwood E. C. & Phillips T. J., 2012). In that study, doses of 10, 20, or 30 mg/kg MOR were administered immediately before a 30 min locomotor activity test. In the MALDR line, the 20 and 30 mg/kg doses of MOR significantly increased locomotor activity, while locomotor activity was not significantly altered by these doses in the MAHDR line. Thus, MOR doses chosen for this drinking procedure were below

doses known to affect locomotor activity. This procedure was repeated on days 6-8. Effects of MOR on consumption of water and 40 mg/l MA was then examined for an additional 4 days. Readings were taken every 2 h to examine time-dependent effects. MOR has an approximately 75-minute half-life in the brain after reaching systemic circulation, and thus, we anticipated that effects on MA intake might be larger earlier during the drinking sessions (Kalvass J. C. *et al.*, 2007). At the end of each 6-h period, the MA tube was removed and the water tube left in place. Mice were weighed every other day and had *ad libitum* access to food and water at all times. The positions of the water and drug tubes were alternated every 2 days, consistent with selection trait testing (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011).

**Effect of MOR on saccharin two-bottle choice drinking**

All experimental details were identical to those used in the MOR-MA two-bottle choice drinking study, except that 0.033% and then 0.066% saccharin was offered vs. water. We have shown that these are preferred concentrations of saccharin (Shabani S. *et al.*, 2011) and that the MADR lines do not differ in consumption of these saccharin concentrations (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). Data were collected in 2 cohorts (final N=8-11/line/dose; N=4-7/line/dose per cohort) and mice were 73-115 days old at the start of the experiment. Only female mice were used for this study, as they were readily available and we did not observe sex effects that interacted with selected line or MOR dose in the MOR-MA study or our previous BUP-MA study (Eastwood E. C. & Phillips T. J., 2014).

**Effects of Fentanyl on MA two-bottle choice drinking**

Data were identically collected in this study except that FEN injections occurred 15 min prior to MA access to account for the shorter half-life of FEN, and mice were placed in a lickometer apparatus each day for 4-h drinking sessions. Mice were female and were 68-86 days of age, and data were collected in 3 cohorts (final n=9-11/line/dose; n=3-4/line/dose/cohort). The lickometer apparatus has been previously described (Eastwood E. C. *et al.*, 2014). Briefly, the chamber is constructed from acrylic plastic, with a wire grid floor and 2,

10-ml drinking tubes with metal sippers. The wire floor and metal sipper tubes create a circuit that is closed when they are simultaneously contacted. MED Associates, Inc. (St Albans, VT) software (MED-PC IV) records sipper contacts, and appetitive (latency to first bout) and consummatory (bout frequency, size, duration, lick rate and interbout interval) variables can be extracted from the cumulative record. The doses of FENT were selected from a previously published FENT locomotor activity study and were doses below those that produced locomotor activation (Eastwood E. C. & Phillips T. J., 2012). After each session, mice were returned to their home cages. Drug dose and position of the MA tube in the lickometer chambers were randomized across mice. MA tube position was held constant because we thought this might reduce variability in the data by eliminating the requirement to relocate the MA-containing tube.

**Membrane preparation**

Female MADR mice were decapitated and brains were extracted. The mPFC and NAc were collected from an approximately ~1-1.3 mm slice of brain tissue which is about 0.5 mm anterior of the anterior commissure. The Vmb was collected from the distal 1.0 mm slice of the hypothalamic region. Regions were removed from the slices using 16 gauge needles. Due to small regions and low protein content, tissue from 5 animals was pooled for each sample. Tissues were placed in 50 mM ice-cold Tris buffer, pH 7.5 and homogenized with a polytron for 30 seconds. Homogenates were centrifuged at 14,000 rpm for 20 min at 4º C. The resulting pellet was washed with ice-cold Tris buffer, pH 7.5, and centrifuged again at 14,000 rpm for 20 min at 4º C. The pellet was resuspended in ice-cold Tris buffer, pH 7.5, and homogenized again with the Polytron. To determine protein concentration, membrane samples were assayed using a BCA protein assay kit (Thermo Scientific, Rockford, IL) with bovine serum albumin as the standard. N=30/line; for a total of N= 6 samples/line (5 regions pooled).

**Ligand binding assay**

Membranes were incubated (2-20 µg protein) with 0.145 - 4.85 nM [$^3$H]DAMGO for the saturation binding assay for 60 min in 50 mM Tris buffer, pH 7.5, in a water bath at 25ºC. Non-

specific binding was measured in the presence of 1 µM unlabeled DAMGO. The incubation was terminated by rapid filtration through Perkin Elmer Filtermat A filters presoaked in 0.05% polyethylenimine on a Tomtec cell harvester. Filters were dried and spotted with scintillation cocktail and radioactivity retained in the filters was counted for 2 min on a Perkin Elmer microBeta plate 1405.

**Two-bottle choice MA drinking in congenics**

Mice were tested using the identical MA drinking procedure that was used to create the MADR lines and described above (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). The number of mice tested was 46 Chr 10 D2.B6 0-20.4 Mb mice (N=9-18/sex/genotype) and 79 Chr 10 D2.B6 0-7.72 Mb mice (N=15-25/sex/genotype). They were 80-140 days of age and data were collected in 7 cohorts of mice (N=13-48 per cohort).

**Statistical analysis**

Data were analyzed by factorial Analysis of Variance (ANOVA) with repeated measures when appropriate, using Statistica software (Statsoft Version 9, Tulsa, OK). Possible independent variables were line, genotype, sex, drug dose or concentration and time. Specific dependent variables analyzed are described under experimental results. Multifactor interactions were examined for the existence of significant two-way interactions within particular levels of a factor and then resolved using simple main effects analysis and the Newman Keuls post-hoc test for mean comparisons, when appropriate. Figures were created using Sigmaplot 2002 for Windows Version 8.0 (SPSS, Chicago, IL). All values are expressed as mean ± standard error of the mean (SEM). The criterion for significance was set at $p<0.05$. Binding data were analyzed using Graphpad Prizm software (version 6.04; San Diego, CA, USA). For saturation curve experiments, the total binding capacity (Bmax) and equilibrium constant ($K_D$) were determined using a one-site model for MOP-r binding sites and compared to Scatchard analysis. Specific

binding was determined by subtracting nonspecific binding from total binding. Comparisons for Bmax and $K_D$ between mouse lines and strains were carried out using the Student's t-test.

**RESULTS**

**Effect of MOR on MA two-bottle choice drinking**

MOR had time- and dose-dependent effects on MA intake. Initial analysis identified significant MA concentration by MOR dose by line [$F(3,121)=2.7$; $p<0.05$], time by MOR dose by line [$F(3,121)=2.9$; $p<0.05$], and MA concentration by time by line [$F(3,121)= 14.0$; $p<0.001$] interactions. No significant sex effects were observed, so data were collapsed on sex for subsequent analyses. Because there were interactions involving MA concentration, data for each concentration were next analyzed separately. For the 20 mg/l concentration, a significant time by dose by line interaction was found [$F(6,242)=3.5$; $p<0.001$]. Each 2-h time block was next examined. There was a significant dose by line interaction during the first 2-h period [$F(3,121)=3.9$; $p<0.5$]. As shown in Fig. 5.2A, there was a significant effect of MOR on MA intake in the MAHDR line ($p<0.01$) and *post hoc* tests indicated that MA intake was significantly lower in MAHDR mice treated with the15 mg/kg dose of MOR, compared to saline-treated MAHDR mice. Additionally, the 10 and 15 mg/kg doses of MOR eliminated the line difference in MA consumption. During the second 2-h period, there was a trend for a dose by line interaction ($p=0.056$) and a significant main effect of line [$F(1,121)=9.6$; $p<0.01$], with the MAHDR consuming more MA compared to the MALDR line (Fig. 5.2B). During the final 2-h period, only a significant line difference in MA intake was found [$F(1,121)=18.3$; $p<0.001$], with no significant effect of MOR (Fig. 5.2C).

For the 40 mg/l concentration, a significant time by line interaction was found [$F(2,242)=16.0$; $p<0.001$], but there were no significant effects of MOR. For each 2-h time period, main effects of line were found [$F(1,121)=6.6$; $p<0.05$ for the first 2 h; $F(1,121)=113.8$; $p<0.001$ for the second 2 h; $F(1,121)=29.7$; $p<0.001$ for the third 2 h] (Fig. 5.2D-F) that supported greater MA consumption in MAHDR than MALDR mice.

Total volume data are summarized in Table 5.1. For total volume of fluid consumed from both the water and MA tubes (ml/kg), significant concentration by dose by sex [($F(3,113)=2.9$; $p<0.05$] and time by dose by sex [$F(3,113)=2.2$; $p<0.05$] interactions were found. There was also a significant concentration by line interaction [$F(1,113)= 12.3$; $p<0.001$]. Data were next examined for each MA concentration. For total volume consumed during the period when the 20 mg/l concentration of MA was available, time by dose and time by sex interactions [$F(6,226)= 2.2$; $p<0.05$ and $F(2,226)=3.2$; $p<0.05$] were detected. Because there were no effects of line, data were next examined for effects of dose and sex, during each time period. During the first two hours, a significant effect of dose was detected [$F(3,125)=7.3$; $p<0.001$]. The 10 and 15 mg/kg doses of MOR reduced total volume consumed, compared to that consumed after saline treatment. No significant dose effects on total volume consumed were detected during the second or third 2-h time blocks. During the second and third 2-h time blocks, there were no significant effects of MOR, but main effects of sex were detected [$F(1,127)= 11.4$; $p<0.001$ and $F(1,127)= 10.2$; $p<0.01$; respectively], with female mice consuming more total volume compared to male mice.

For total volume consumed during the 40 mg/l MA concentration access period, significant time by dose [$F(6,226)=2.5$; $p<0.05$] and time by sex [$F(2,2260= 7.7$; $p<0.005$] interactions were found. Again, data were examined for effects of dose and sex, during each time period. There was a significant effect of dose only during the first 2-h period [$F(3,125)=5.0$; $p<0.01$]. The highest MOR dose (15 mg/kg) reduced total volume intake compared to intake in saline-treated animals. During the next 2, 2-h periods there were significant effects of sex [$F(1,127)=9.2$; $p<0.01$ and $F(1,127)=6.3$; $p<0.05$], with female mice consuming more in total volume per body weight, compared to male mice.

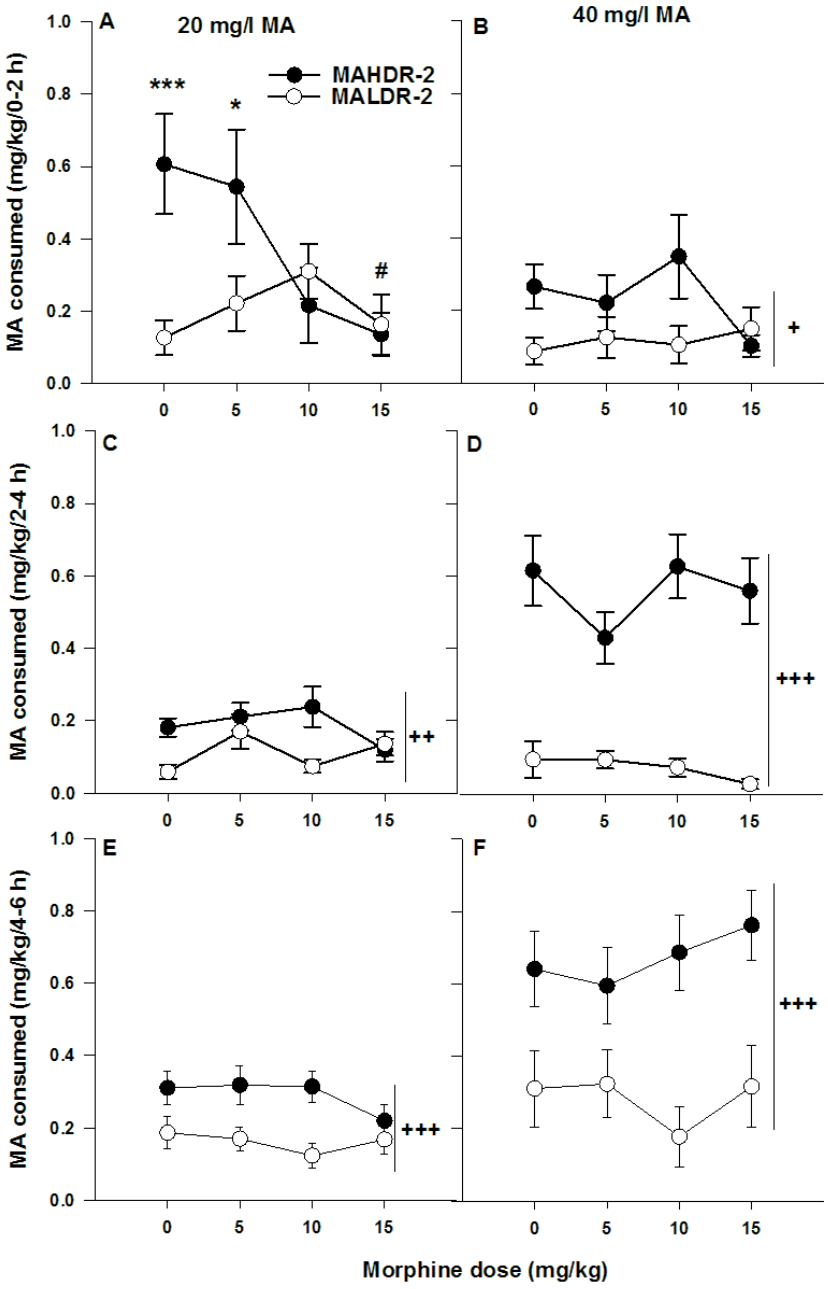


**Figure 5.2. Higher doses of morphine reduce MA intake**. Shown are means ± SEM for 20 mg/l MA consumption (A-C) and 40mg/l MA consumption (D-F) for three 2-h periods of a 6-h MA vs. water limited access study. MADR mice were treated with saline or one of several doses of morphine 30 min prior to each 6-h MA vs. water access period, which occurred during the first 6 h of the dark phase of the light:dark cycle. Each data point is a 2-day average for days 2 and 4 of a 4-day period for each MA concentration. *P <0.05, **P <0.01 and ***P <0.001 for the line

difference at the indicated morphine dose. #P <0.05 for the difference between the saline (0 dose) and morphine dose mean. +P<0.05, +++P <0.001 for the main effect of line. Final N =15-18/line/dose.

**Table 5.1: Effects of morphine on total volume consumed during a MA vs water two-bottle choice drinking procedure.**

| Line | Dose | 20 mg/l MA 0-2 h (ml/kg) | 20 mg/l MA 2-4 h (ml/kg) | 20 mg/l MA 4-6 h (ml/kg) | 40 mg/l MA 0-2 h (ml/kg) | 40 mg/l MA 2-4 h (ml/kg) | 40 mg/l MA 4-6 h (ml/kg) |
|---|---|---|---|---|---|---|---|
| MAHDR | 0 mg/kg MOR | 17.5 ± 2.0 | 27.2 ± 3.7 | 36.9 ± 3.2 | 24.7 ± 4.1 | 41.0 ± 4.4 | 44.8 ± 4.2 |
| MALDR | 0 mg/kg MOR | 24.3 ± 8.3 | 28.4 ± 4.1 | 41.2 ± 5.1 | 19.3 ± 3.9 | 41.0 ± 4.4 | 43.7 ± 6.8 |
| MAHDR | 5 mg/kg MOR | 13.4 ± 2.6 | 30.9 ± 2.7 | 36.1 ± 4.0 | 21.5 ± 4.0 | 34.1 ± 5.0 | 50.5 ± 3.4 |
| MALDR | 5 mg/kg MOR | 14.9 ± 2.5 | 38.8 ± 4.4 | 40.3 ± 4.5 | 17.3 ± 2.8 | 39.0 ± 4.4 | 45.0 ± 4.4 |
| MAHDR | 10 mg/kg MOR | 8.6 ± 1.5* | 28.5 ± 4.0 | 38.3 ± 3.8 | 22.1 ± 4.7 | 38.5 ± 4.8 | 46.8 ± 5.2 |
| MALDR | 10 mg/kg MOR | 11.9 ± 2.6* | 28.0 ± 3.6 | 36.9 ± 6.2 | 15.5 ± 3.5 | 45.8 ± 3.9 | 36.3 ± 5.4 |
| MAHDR | 15 mg/kg MOR | 3.4 ± 1.0+ | 16.3 ± 1.7 | 27.5 ± 3.3 | 5.9 ± 1.4+ | 31.2 ± 3.6 | 39.4 ± 3.3 |
| MALDR | 15 mg/kg MOR | 3.7 ± 1.3+ | 27.1 ± 2.7 | 32.1 ± 4.4 | 12.5 ± 2.8+ | 33.3 ± 5.0 | 42.6 ± 5.3 |

Shown are mean ± SEM for total volume consumed (ml/kg) during the 6-h limited access two bottle choice MA vs water drinking study, in 2-h time periods. Pretreatments of 0, 5, 10, or 15 mg/kg MOR were given 15 min before beginning the MA access period. The first row lists the MA concentration that was offered vs. water at the time total volume was assessed. *$p<.05$ for the effect of the 10 mg/kg MOR dose (collapsed on line), compared to the 0 mg/kg MOR dose (collapsed on line); +$p<.001$ for the effect of the 15 mg/kg MOR dose (collapsed on line), compared to the 0 mg/kg MOR dose (collapsed on line)

**Effect of MOR on saccharin two-bottle choice drinking**

Morphine had significant time- and dose-dependent effects on saccharin intake and total volume consumed. Initial analyses identified a significant 4-way interaction of concentration by time by dose by line [$F(4,110)=2.9$; $p<0.05$]. For consistency with the analysis of data from the MOR-MA study, we examined data for each saccharin concentration. For 0.033% saccharin (Fig. 5.3A-C), only significant main effects of dose [$F(2,58)=4.4$; $p<0.05$] and time [$F(2,116)=10.5$; $p<0.001$] were detected. Post-hoc analysis indicated that the 15 mg/kg MOR dose reduced saccharin intake regardless of selected line ($p<0.05$) and there was more saccharin consumed during the third, 2-h period than during the first 2, 2-h periods ($p<0.001$, $p<0.01$; respectively). For the 0.066% saccharin concentration (Fig. 5.3D-F), only a significant main effect of time was detected [$F(2,110)=4.2$; $p<0.05$]. Post-hoc analysis indicated that more saccharin was consumed during the final 2-h period, compared to the first 2-h period ($p<0.05$).

Total volume data are summarized in Table 5.2. For total volume consumed (ml/kg), initial analyses identified significant concentration by line [$F(1,55)=6.7$; $p<0.05$], concentration by time [$F(2,110)=42.4$; $p<0.001$], and time by dose [$F(4,110)=4.4$; $p<0.001$] interactions. For the 0.033% saccharin concentration, a significant time by dose interaction was detected [$F(4,110)=4.2$; $p<0.01$]. Follow-up analyses identified a significant main effect of dose for the first 2-h time period [$F(2,55)=7.0$; $p<0.01$], but not the later time periods. The 15 mg/kg dose of MOR reduced saccharin consumed, during the first 2 h, compared to saline-treated mice. For the 0.066% saccharin concentration, significant effects of dose [$F(2,55)= 3.2$; $p<0.05$] and time [$F(2,110)= 6.0$; $p<0.01$] were detected. Follow-up analyses determined that the 15 mg/kg MOR dose significantly reduced total volume consumed and that more saccharin was consumed during the second 2-h period.

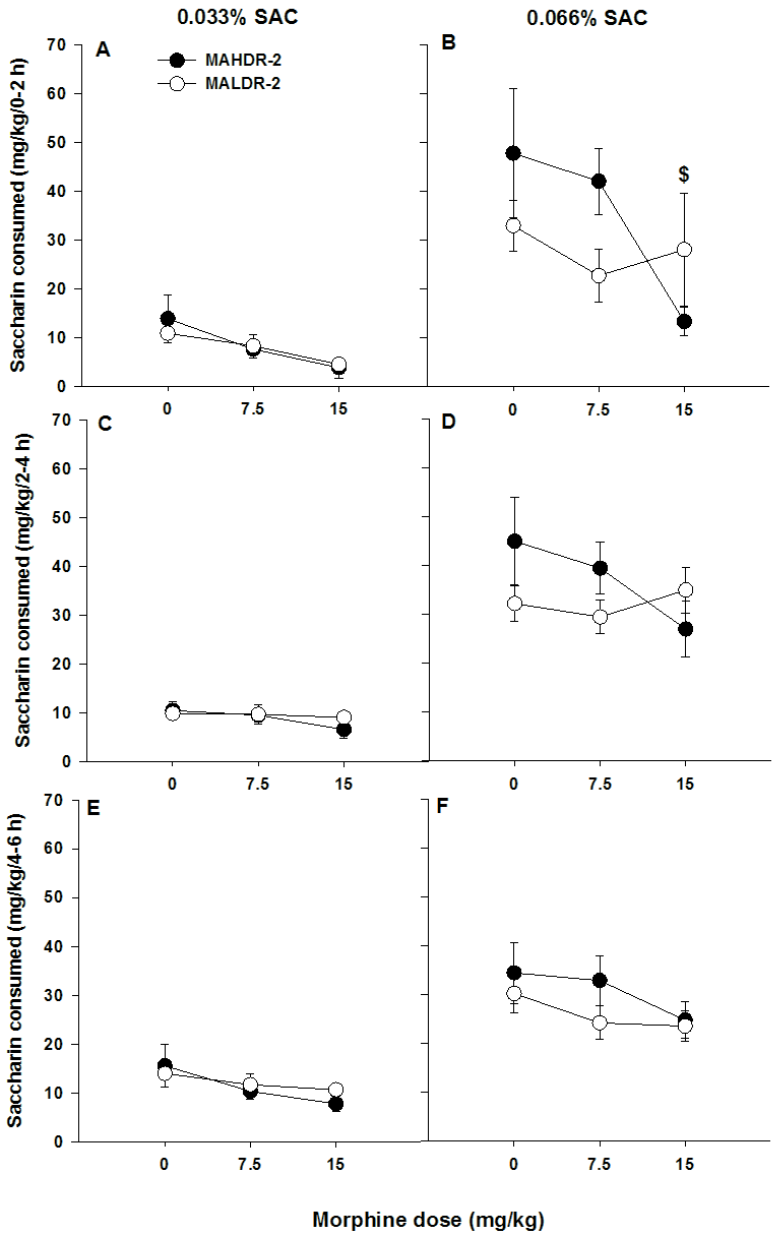


**Figure 5.3. High doses of morphine reduce saccharin intake.** Shown are mean ± SEM 0.033% (A-C) and 0.066% (D-F) saccharin consumed for three 2-h periods of a 6-h saccharin vs. water limited access study. Mice were treated with saline or one of several doses of morphine, 30 min prior to each 6-h access period during the dark phase of the light:dark cycle. Each data point is a 2-day average for days 2 and 4 of a 4-day drinking period for each

saccharin concentration. $P<0.05 for the main effect of dose, indicating a difference between saline (0) and the 15 mg/kg dose of morphine. Final N =8-11line/dose.

**Table 5.2: Total volume consumed during a saccharin vs water two-bottle choice drinking procedure.**

| Line | Dose | 0.33% SAC 0-2 h (ml/kg) | 0.33% SAC 2-4 h (ml/kg) | 0.3% SAC 4-6 h (ml/kg) | 0.66% SAC 0-2 h (ml/kg) | 0.66% SAC 2-4 h (ml/kg) | 0.66% SAC 4-6 h (ml/kg) |
|---|---|---|---|---|---|---|---|
| MAHDR | 0 mg/kg MOR | 33.0 ± 3.6 | 41.6 ± 4.5 | 61.5 ± 13.2 | 76.2 ± 19.1 | 76.4 ± 12.2 | 60.6 ± 7.2 |
| MALDR | 0 mg/kg MOR | 25.3 ± 2.8 | 40.4 ± 4.8 | 65.0 ± 6.8 | 53.7 ± 7.5 | 56.1 ± 5.4 | 55.7 ± 4.8 |
| MAHDR | 7.5 mg/kg MOR | 20.5 ± 8.0 | 42.3 ± 4.0 | 44.6 ± 3.8 | 70.5 ± 10.2 | 69.3 ± 7.7 | 58.0 ± 7.4 |
| MALDR | 7.5 mg/kg MOR | 23.5 ± 3.7 | 44.3 ± 4.8 | 48.3 ± 5.7 | 42.8 ± 7.6 | 58.6 ± 4.2 | 45.2 ± 4.3 |
| MAHDR | 15 mg/kg MOR | 5.1 ± 1.1+ | 27.0 ± 5.6 | 50.2 ± 7.4 | 22.1 ± 4.7+ | 46.8 ± 8.5 | 46.8 ± 8.5 |
| MALDR | 15 mg/kg MOR | 15.2 ± 5.2+ | 43.8 ± 4.6 | 58.2 ± 4.6 | 46.0 ± 17.3+ | 64.1 ± 7.9 | 64.1 ± 7.9 |

Shown are mean ± SEM for total volume consumed during the 6-h limited access two-bottle choice saccharin (SAC) vs water drinking study. Pretreatments of 0, 7.5 or 15 mg/kg MOR were given 15 min before beginning the SAC access period. The first row lists the SAC concentration that was offered vs. water at the time total volume was assessed. +p<.001 for the effect of the 15 mg/kg MOR dose (collapsed on line), compared to the 0 mg/kg MOR dose (collapsed on line)

**Effects of Fentanyl on MA two-bottle choice drinking**

Data for this study were recorded on every day of MA access, but results are given here for the fourth day on which each concentration of MA was offered. These data are representative of the overall results and allow for direct comparison to data shown for the same time period, in which we examined patterns of MA intake in the MADR mice. In that analysis, stronger correlations were found between cumulative licks and volume consumed for the day 4 measure (Eastwood E. C. *et al.*, 2014). When both concentrations of MA were included in the overall analysis, significant main effects of line [$F(1,46)= 24.9$; $p<0.001$] and of MA concentration [$F(1,46)= 6.1$ $p<0.05$] were found. MAHDR mice consumed significantly more MA, compared to MALDR mice, as expected (Fig. 5.4A and B). However, there was no significant effect of FENT pretreatment on the amount of MA consumed, and the selected lines did not differ in total volume (ml/kg) consumed (Fig. 5.4C and D).

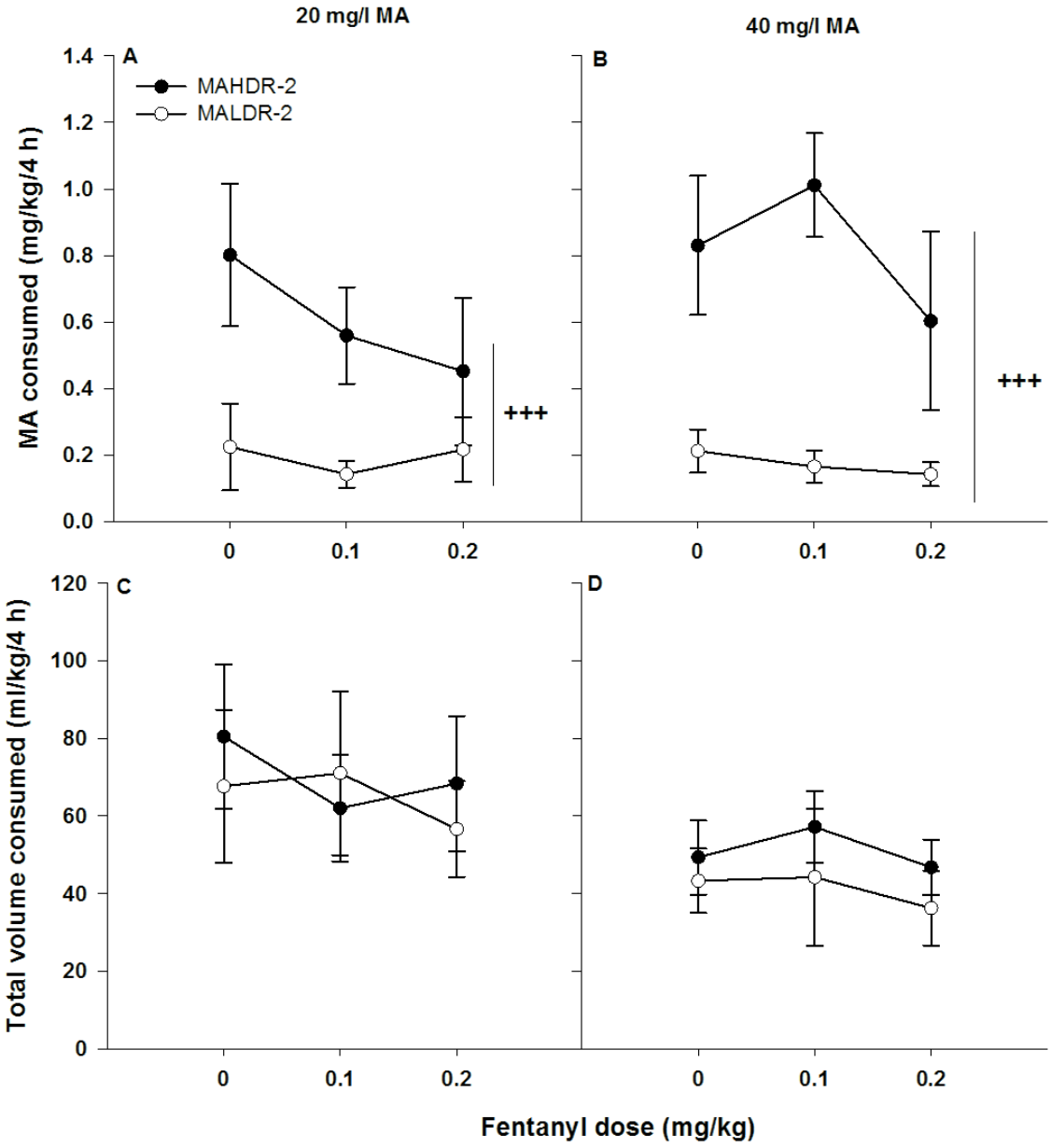


**Figure 5.4. Consumption of MA and total volume during a 4-h limited access two-bottle drinking session in a lickometer apparatus.** Shown are mean ± SEM mg/kg consumption of MA from either a 20 (A) or 40 (B) mg/l solution, and the corresponding total volume consumed (C and D) in MAHDR-2 and MALDR-2 mice. Mice were pretreated with saline, 0.1 or 0.2 mg/kg of fentanyl immediately before a 4-h limited access session to MA vs water during the dark phase of the light:dark cycle. +++$p<0.001$ for the main effect of line. Final N=7-10/dose/line.

**Effect of Fentanyl Pretreatment on Lick Pattern and Total Licks**

Although there were no effects of FENT on the amount of MA consumed, FENT has a short half-life and its transient effects might be detected earlier during the drinking session. As the lickometer device records cumulative licks, reductions in the total number of licks or number of MA bouts would indicate potential effects of FENT on MA intake. For total number of licks from the MA-containing tube, during the entire 4-h session, a significant main effect of line [$F(1,46)= 7.8$; $p<0.01$] and a concentration by dose interaction [$F(2,46)= 3.7$; $p<0.05$] were found. MAHDR mice took more licks from the MA-containing tube, compared to MALDR mice. Because there was a significant concentration by dose interaction, effects of FENT for each concentration were examined. For the 20 mg/l MA solution (Fig. 5.5A), a significant line by dose interaction was found [$F(2,46)=4.8$; $p<0.05$]. Compared to MALDR mice, MAHDR mice had more licks from the MA-containing bottle following the 0 and 0.1 mg/kg FENT pretreatments ($p<0.05$ and $p<0.001$, respectively), but there was no line difference following treatment with the 0.2 mg/kg FENT dose. No dose or line effects were detected for total licks from the water bottle offered during the 20 mg/l MA session (Fig. 5.5B). For the 40 mg/l MA solution (Fig. 5.5C), there was only a significant a main effect of line [$F(1,45)=8.3$; $p<0.01$], with MAHDR mice taking more licks, compared to MALDR mice. No significant dose or line effects were detected for total licks from the water bottle during the 40 mg/l MA session (Fig. 5.5D).

Data were next separated into 30-min intervals and examined for time-dependent effects of FENT. This analysis is consistent with our previously published lickometer evaluation of MA drinking data (Eastwood E. C. *et al.*, 2014). No significant main or interaction effects of dose were detected when the entire 4-h session was examined in 30 min bins (data not shown). However, the initial 30 min bin was next examined separately to detect any short-lasting effects of FENT (Fig. 5.6). A significant main effect of dose was detected during the first 30 min [$F(2,49)=5.2$; $p<0.01$], that was not dependent upon concentration. The 0.2 mg/kg dose reduced licks taken from the MA-containing bottle compared to number of licks in saline-treated mice

(Fig. 5.6A and B). Analysis of licks from the water-containing bottle during the first 30 min also identified a significant main effect of dose [$F(2,49)=3.9$; $p<0.05$], with the 0.2 mg/kg FENT dose significantly reducing licks from the water-containing bottle (Fig. 5.6C and D).

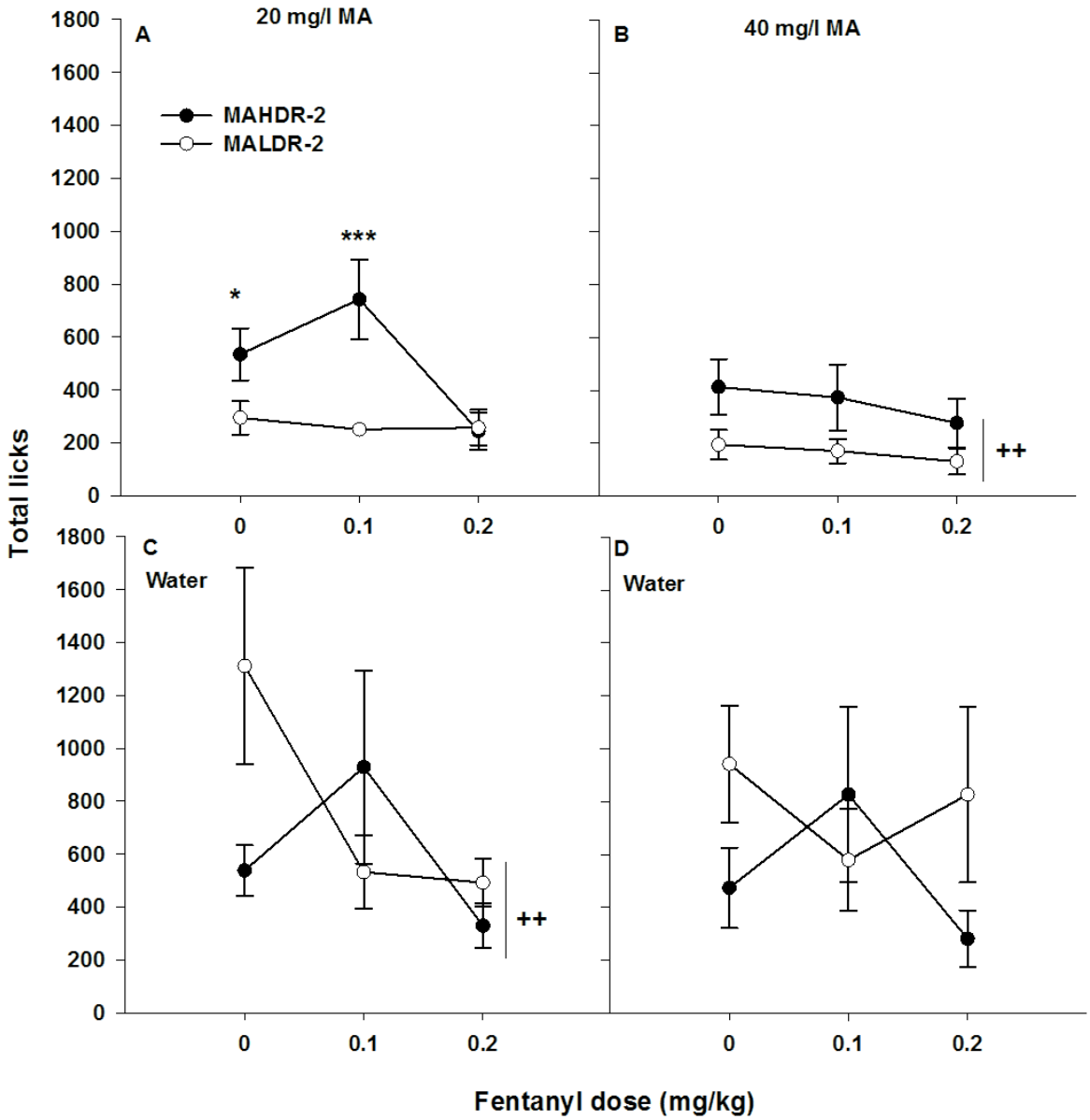


**Figure 5.5: Consumption of MA and total volume during a 4-h limited access two-bottle drinking session in a lickometer apparatus.** Shown are mean ± SEM total licks from either the 20 (A) or 40 (B) mg/l MA solution and corresponding water bottle offered (Panels C and D) in MAHDR and MALDR mice. Mice were pretreated with either saline or 0.1 or 0.2 mg/kg of fentanyl immediately before a 4h limited access session to MA vs water during the dark phase of the light:dark cycle. *p<0.05, ***p<0.001 for the line difference at the indicated dose. ++p<0.01 for the main effect of line. Final N=7-10/dose/line.

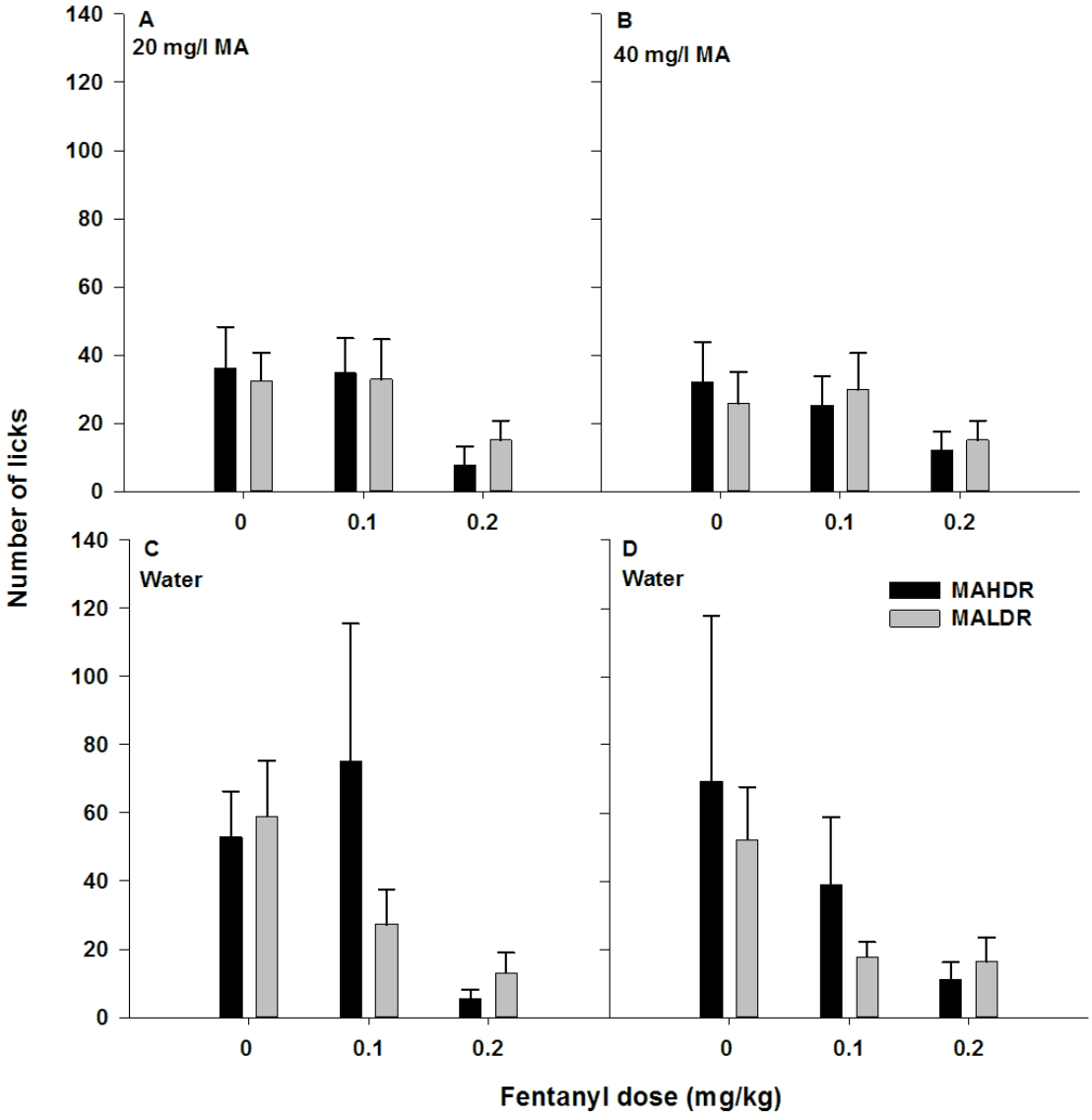


**Figure 5.6:** Shown are mean ± SEM licks from the MA-containing bottle across the first 30 min of a 4-h session for MAHDR and MALDR mice on day 4 access of either the 20 mg/l (A), or the 40 mg/l (B) MA concentration, and from the corresponding water-containing bottle (C and D). There were no concentration-dependent effects, but data are shown for each concentration for comparison to other data. There were significant effects of the 0.2 mg/kg FENT dose on licks from the MA and water tubes, regardless of line or MA concentration (no significance symbols shown, because these were main effects). Final N=7-10/dose/line.

**Effect of Fentanyl Pretreatment on Bout Patterns**

There were no significant effects of FENT dose or difference between the MADR lines for latency to first drinking bout. Additionally, there were no significant effects of line or dose on licks in the first bout, first bout length, or first bout rate. In combination, the first bout, the licks, and the drinking data showed transient effects of FENT on MA intake and did not support additional analysis of other bout characteristics. Analysis of bout characteristics from the water bottle when the 20 and 40 mg/l MA solutions were offered, found no significant effects of mouse line or dose for latency to first bout, licks in first bout, first bout length, or first bout rate. Due to the absence of any significant effects, data are not shown.

**[$^3$H]DAMGO binding**

Binding data are summarized in Table 5.3. Mu-opioid receptors were identified in membrane homogenates of mPFC, NAc, and Vmb brain tissue by saturation binding of the MOP-r ligand, [$^3$H]DAMGO. The MADR lines did not significantly differ in maximal number of receptors (Bmax) in the NAc or Vmb regions; however, MALDR mice had a significantly greater number of receptors in the mPFC compared to MAHDR mice [$t(12)=3.98$; $p<0.001$]. There were no differences in the affinity ($K_D$) for DAMGO in mPFC, NAc, or Vmb between the MADR lines of mice. There were no differences in the number of receptors or in the affintity for DAMGO in the mPFC, NAc, or Vmb regions between B6 and D2 mice.

**Table 5.3: MOP-r density and affinity in MADR, B6 and D2 mice.**

| Region | Line/Strain | Bmax (fmol bound/mg protein) | KD (nM) |
|---|---|---|---|
| mPFC | MAHDR | 65.7 ± 18.3*** | 1.5 ± 1.0 |
| | MALDR | 152.7 ± 54.8 | 2.8 ± 2.0 |
| | B6 | 48.57 ± 11.09 | 1.30 ± 0.75 |
| | D2 | 53.07 ± 9.49 | 1.63 ± 0.69 |
| NAc | MAHDR | 337.5 ± 119.0 | 3.0 ± 2.1 |
| | MALDR | 366.0 ± 108.5 | 2.6 ± 1.6 |
| | B6 | 176.2 ± 29.84 | 1.24 ± 0.54 |
| | D2 | 254.4 ± 52.28 | 4.11 ± 1.48 |
| Vmb | MAHDR | 262.9 ± 51.1 | 2.5 ± 1.0 |
| | MALDR | 345.0 ± 109.4 | 4.7 ± 2.5 |
| | B6 | 374.4 ± 133.9 | 3.05 ± 2.11 |
| | D2 | 541.3 ± 107.4 | 3.84 ± 1.37 |

Values were determined using concentration of [$^3$H]DAMGO from 0.145-4.85 nM as described in the methods section. Each value represents the mean ± SEM of tissues from 5-7 samples (5 animals pooled for each), all performed in duplicate. ***p<0.001, for the comparison between MAHDR and MALDR. All saturation curves were best fit using a one-site model.

**MA Drinking in Chr 10 D2.B6 (0-7.72) and Chr 10 D2.B6 (0-20.4) congenic and D2 mice**

No sex-dependent effects were detected in any of the initial analyses for MA intake or total volume consumed for the congenic and background strains. As shown in Fig. 5.7A, there were no genotype-dependent effects detected for MA intake between the Chr 10 D2.B6 (0-7.72) congenic strain and the background D2 strain. A main effect of MA concentration was detected, [$F(1,78)=82.1$; $p<0.001$], with greater MA consumed at the higher concentration of MA. For total volume consumed (ml/kg) during the time that the two concentrations of MA were offered, there were no significant effects of genotype (Fig. 5.7B). A main effect of MA concentration was detected [$F(1,78)=30.1$; $p<0.001$], with greater total volume consumed when the higher concentration of MA was offered. For the Chr 10 0-20.4 Mb strain versus the D2 background strain, there was a significant genotype by MA concentration interaction [$F(1,43)=7.4$; $p<0.05$] for MA consumption (Fig. 5.7C). For both concentrations of MA, the congenic strain consumed significantly less MA, compared to the background strain. For total volume consumed (ml/kg), there was no significant effect of genotype. A main effect of concentration was detected [$F(1,43)=12.1$; $p<0.01$], with greater total volume consumed when the higher concentration of MA was offered (Fig. 5.7D).

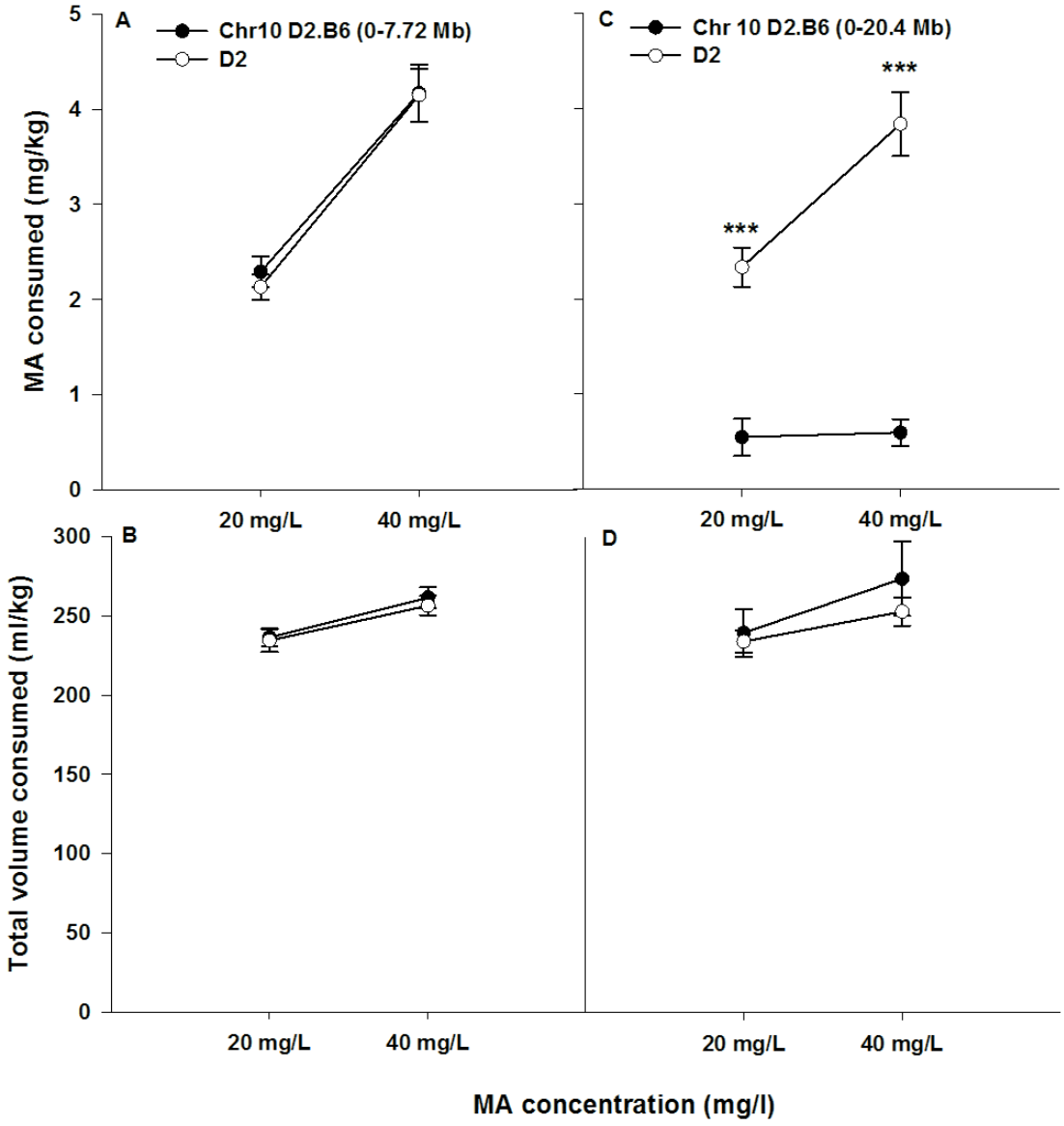


**Figure 5.7. MA Drinking in Chr 10 D2.B6 (0-7.72) and Chr 10 D2.B6 (0-20.4) congenic and D2 mice.** Data shown are (A and C) average MA consumed (mg/kg) on the second and fourth days of access to the 20 mg/l and 40 mg/l MA solutions and (B and D) average total volume consumed on the same days, corrected for body weight (ml/kg). All values are expressed as mean ± SEM for the DBA2/J (D2) and congenic mice (Chr 10 D2:B6 0-7.72 Mb) in panels A and B and for the D2 and congenic mice (Chr 10 D2.B6 0-20.4 Mb) in panels C and D. ***p<0.001 for the genotype difference at the indicated concentration. Final N=6-10/strain/sex/genotype for

the D2:B6 Chr 10 0-7.58 Mb and D2 mice, and 15-25/strain/sex/genotype for the Chr 10 D2.B6 0-20.4 Mb and D2 mice.

## DISCUSSION

The MOP-r agonist drug, MOR, reduced MA intake in the MAHDR line; however, saccharin intake and total volume consumed were also reduced, regardless of selected line. line. MOR reduced intake of all fluids, potentially by inducing a behavior that affected the ability to perform drinking behavior. The MOP-r agonist drug, FENT, did not significantly reduce total MA intake in a 4-h period. It did reduce the number of licks from the MA-containing bottle, but the highest dose also decreased the number of licks from the water-containing bottle, regardless of selected line. This suggests that FENT had non-specific effects on fluid intake. The two Chr 10 congenic strains did not show congruent results for MA drinking vs. the D2 background strain. Only the congenic strain with the larger Chr 10 B6 segment exhibited decreased MA intake, compared to the background strain. Because *Oprm1* was in both introgressed congenic segments, these data eliminate *Oprm1* as a quantitative trait gene.

Although there were some non-specific effects on fluid intake, the MOP-r agonist drugs had larger dose-dependent effects on MA intake when the 20 mg/l MA solution was offered than when the 40 mg/l MA solution was offered. This might have been due to the development of tolerance to MOP-r drug effects with repeated exposure, since our procedure used a consecutive testing protocol for 20 then 40 mg/l MA. In another study, evaluation of the intermittent dosing of either MOR (13.4 – 27.6 mg/kg) of FENT (0.006 – 0.66 mg/kg) every 24 h for either 3 or 7 consecutive doses demonstrated that 3 days of exposure was sufficient for tolerance to develop and produce decreases in the antinociceptive potencies of MOR and FENT in mice (Duttaroy A. & Yoburn B. C., 1995). Duttaroy and Yoburn (1995) also showed that MOP-r agonist drugs with different pharmacological profiles produced the same magnitude of tolerance. Our protocol used 8 intermittent injections spaced 24 h apart, with 4 of those occurring prior to access to the 40 mg/l MA concentration after 4 prior treatments. Thus, the

reduced effectiveness could be related to the development of tolerance. However, there is some evidence that drugs with lower efficacy for the MOP-r, such as MOR, produce greater tolerance than drugs with higher MOP-r efficacy, such as FENT. One study found that a single microinjection of MOR into the ventrolateral periaqueductal gray of the rat produced a rightward shift of the dose-response curve on a thermal hotplate assay, while 4 to 8 consecutive microinjections of FENT were required to cause an equivalent rightward shift in the dose-response curve (Jones D. N. & Holtzman S. G., 1992; Bobeck E. N. *et al.*, 2012). Although, these studies demonstrate greater tolerance with lower efficacy MOP-r agonist drugs, MOR and FENT had similar effects on MA intake and total volume when either MA solution was offered, suggesting that tolerance to a MOP-r agonist drug likely did not impact drinking in MADR mice.

An alternative explanation to the development of tolerance is that MAHDR mice have greater drive to consume the 40 mg/l than the 20 mg/l MA concentration. However, a comparison of MA preference ratio in MAHDR mice for the 20 mg/l vs 40 mg/l MA concentration does not support this alternative hypothesis. MAHDR mice have significantly greater preference ratios for MA compared to MALDR at both concentrations offered, but have a slightly greater preference for the 20 mg/l solution compared to the 40 mg/l solution (PR: 0.53 ± 0.02 vs 0.59 ± 0.02 for MAHDR; 0.07 ± 0.01 vs 0.04 ± 0.01 for MALDR) (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011).

Although pretreatment with MOR produced significant dose-dependent decreases in MA intake during the 6-h limited access drinking session, decreases in total volume were also observed. Our previously published data examining the ability of the MOP-r partial agonist drug, BUP, to reduce voluntary MA intake, found that low doses (1 and 2 mg/kg) reduced MA intake during the first 4 h of a 6-h session, when a 20 mg/l MA solution was offered, and during the first 2 h of a 6-h session when a 40 mg/l MA solution was offered (Eastwood E. C. & Phillips T. J., 2014). BUP did not have significant overall effects on fluid consumption. Because MOR reduced total volume consumed, we performed an additional experiment examining the effect of MOR

pretreatment on saccharin vs water consumption to see if the same outcome would be obtained. During the first 2 h of the 6-h session, decreases in saccharin and total volume consumed at both saccharin concentrations were observed. Although it is possible that MOR had an impact on the desire to consume MA and saccharin via interactions with reward mechanisms, it seems likely that behavioral effects of MOR may have interfered with drinking behavior.

As determined using cloned opioid receptors, FENT has nearly exclusive activity at MOP-r and has the greatest binding potency (Ki) at the MOP-r subtype, compared to δ, and κ opioid receptors, while MOR has high affinity for both the MOP-r and κ-receptor subtypes (Raynor K. *et al.*, 1994). Data in mice indicate that following a single subcutaneous bolus dose of MOR, the drug was absorbed into blood tissue in 0.55 min and had approximately a 74 min half-life. FENT on the other hand was absorbed more quickly (0.07 min) and had approximately a 4.9 min half-life in brain (Kalvass J. C. & Pollack G. M., 2007). There is high concordance between the pharmacokinetics of acutely administered MOR and FENT between rodents and humans. These pharmacological profiles are consistent with FENT being more lipophilic than MOR and reaching target tissues at a more rapid rate. Our data show that MOR produced time-dependent effects in the MOR-MA and MOR-SAC drinking studies during the first 2 h of the 6-h limited access drinking session, which is consistent with a half-life of a little over an hour. With regard to our other published data examining BUP treatment effects on MA intake, BUP and FENT are both more potent and lipophilic MOP-r agonist drugs compared to MOR. Further, BUP takes longer to dissociate from the MOP-r compared to FENT (166 vs 7 min, respectively) (Boas R. A. & Villiger J. W., 1985).

In the ethanol literature, there is strong evidence to support reductions in both ethanol and saccharin intake following MOP-r antagonist drug pretreatment, while MOP-r agonist drugs have been shown to enhance ethanol and saccharin intake (Hubbell C. L. *et al.*, 1986; Volpicelli J. R. *et al.*, 1991; Zhang M. & Kelley A. E., 2002). However, high doses of MOR (30 mg/kg) were shown to reduce ethanol intake (Sinclair J. D., 1974). The doses of MOR used here were

well below this high dose of 30 mg/kg (5, 7.5, 10, and 15 mg/kg) and were selected because they were below doses that produced locomotor stimulation in MALDR mice during a previous acute locomotor activity study where MADR mice received 0, 10, 20, or 30 mg/kg MOR (Eastwood E. C. & Phillips T. J., 2012). Although no dose of MOR had significant locomotor effects in MAHDR mice, MA intake was decreased in mice of this line. However, MOR also reduced saccharin and total volume consumed. Again, MOR may have produced increases in an unknown competing behavior, which interfered with the animals' ability to engage in drinking behavior. FENT also had several dose-dependent effects in the lickometer study. The doses selected for this study (0.1 and 0.2 mg/kg) did not have significant effects on locomotor activity in the MAHDR mice (Eastwood E. C. & Phillips T. J., 2012). However, FENT decreased the number of licks from the MA-containing and water-containing bottles during the first 30 min of the 4-h session. Despite this, no significant effects on total volume intake or drinking microstructure were detected. As discussed, FENT has a short half-life and volume data were collected only for the entire 4-h period, which was likely not sensitive to changes that may have occurred early in the session and that could have been masked by rebound drinking later in the session.

Our [$^{3}$H]DAMGO binding data are consistent with greater expression of *Oprm1* in the mPFC of MALDR mice, resulting in greater protein expression. They are also consistent with the absence of an *Oprm1* expression difference between the selected lines in the NAc and Vmb (Belknap J. K. *et al.*, 2013). The PFC is the major source of glutamate projections to the NAc and Vmb (Sesack S. R. *et al.*, 1989; Reid M. S. *et al.*, 1997; Carr D. B. & Sesack S. R., 2000b; Gulley J. M. & Stanis J. J., 2010). Application of the MOP-r agonist, DAMGO, into the mPFC attenuated glutamate-induced mPFC neuron firing, which was reversed by naloxone (Giacchino J. L. & Henriksen S. J., 1998). MA has been shown to increase levels of endogenous opioids (Schad C. A. *et al.*, 2002) and greater MOP-r activity in the mPFC after MA administration could reduce the activity of mPFC glutamate projections onto GABA-containing neurons in the NAc,

and ultimately reduce DA levels. Reduced tonic DA levels in the MALDR mice could have an aversive effect or reduce the rewarding effects of MA. We speculate that a larger number of MOP-r in MALDR mice in the mPFC leads to greater attenuation of glutamate neuron firing when MA is consumed or administered in this line than in the MAHDR line. This difference could lead to differential levels of DA in the NAc, leading to differences in the perceived rewarding properties of MA between the selected lines.

Based on the data from the MALDR and MAHDR mice, it was hypothesized that the B6 strain would have greater MOP-r density in the mPFC region compared to D2 mice, and that the strains would have similar levels of MOP-r binding in the NAc and Vmb regions. Instead, no strain differences in MOP-r density were found in any of these brain regions. That a difference was not observed between B6 and D2 mice in the mPFC, but was observed between MALDR and MAHDR mice, indicates that the selected lines do not resemble their progenitor strains for all traits examined and that the difference in mPFC MOP-r density could be a product of selection for low vs high levels of MA intake.

The congenic MA drinking data indicate that the 12.86 Mb non-overlapping B6 segment on Chr 10 between 7.58 and 20.4 Mb, contains a gene that reduces MA intake on a D2 strain background. Both congenic strains contained *Oprm1* (located at 6.75 Mb) in their introgressed B6 segment, and we have previously demonstrated that D2 mice consume more MA compared to B6 mice and that D2 alleles in the Chr 10 QTL are associated with greater MA intake (Eastwood E. C. & Phillips T. J., 2012; Belknap J. K. *et al.*, 2013). It was hypothesized that *Oprm1* influences MA intake, and therefore, that both congenics would consume less MA compared to the D2 background strain. The fact that we did not observe a reduction in both congenics, suggests that *Oprm1,* is not a quantitative trait gene contributing to the genetic variance in MA intake. These studies were initiated prior to the publication of a gene network analysis of transcriptionally active genes between the MADR lines that also did not support *Oprm1* as a quantitative trait gene. However, that study did find that *Oprm1* is modulated by

other transcriptionally active genes in the network relevant to risk for MA drinking between the MAHDR and MALDR lines (Belknap J. K. *et al.*, 2013). In the 12.86 Mb non-overlapping region, there are approximately 55 protein coding genes. Additional fine mapping of this interval would be beneficial for reducing the number of putative candidate genes to be considered for their influence on genetic risk for MA intake. One approach to finer mapping would be to create additional interval-specific congenic strains by backcrossing the existing congenic that captured the MA drinking trait to the D2 background strain. Then animals with smaller relevant B6 segments would be identified by genotyping and new congenics for smaller segments created and tested for MA intake.

Based on previously collected microarray expression data between the 2 replicates of MADR line mice, 231 genes or gene transcripts on Chr 10 were DE. Of these, 30 are protein coding genes that reside in the 12.86 non-overlapping segment between 7.58 and 20.4 Mb on mouse Chr 10 (Belknap J. K. *et al.*, 2013). A couple of potentially interesting candidates within this interval include the glutamate metabotropic receptor 1 gene, *Grm1*, and the neuromedin B receptor gene, *Nmbr*. *Nmbr* plays a role in the perception of thermal pain, regulating body temperature, and stress behavioral responses (Ohki-Hamazaki H. *et al.*, 1999; Mishra S. K. *et al.*, 2012). MA has differential effects on body temperature in MAHDR and MALDR mice (Harkness et al., unpublished) and *Nmbr* could have a role. Glutamate group 1 metabotropic receptors are expressed in reward regions of the brain, including the VTA, NAc, and PFC (Kenny P. J. & Markou A., 2004). Inhibition of glutamate metabotropic group 1 receptors attenuated MA-induced locomotor activity and Grm1 knockout mice had increased AMPH-induced locomotor activity and did not show AMPH-induced increases in prodynorphin, compared to wild type mice (Mao L. *et al.*, 2001; Satow A. *et al.*, 2008).

In summary, our congenic MADR data rules *Oprm1* out as a gene that influences genetic risk for MA intake in the MAHDR and MALDR selectively bred mouse lines. Furthermore, although MOR and FENT reduced MA intake and/or licks from the MA-containing

bottle in a dose-dependent manner in MAHDR mice, reductions in saccharin and total fluid intake were also observed. These data suggest that the MOP-r agonists did not specifically engage a mechanism involved in MA intake, but rather that a competing behavioral effect of the MOP-r agonist drug interfered with drinking ability. However, these data are not consistent with the specific effects of a partial agonist on MA intake, which could have greater promise as a treatment agent or could have been acting through non-opioid mechanisms. Assessment of MOP-r binding (Bmax and affinity) in consistent with previous gene expression data, and supports a difference in MOP-r density in the mPFC in MALDR, compared to MAHDR mice. However, MOP-r binding assessment in B6 and D2 mice did not reveal strain differences that resemble the patterns seen in the MADR lines. The drinking and congenic data do not completely rule out opioid involvement in MA intake, as *Oprm1* might be modulated by other gene that influence the difference in risk for MA intake between the selected lines.

## CHAPTER 6: General Discussion

Though many genes reside within the ~40 Mb confidence interval of a QTL for MA drinking identified on Chr 10 in MADR mice, I chose for my dissertation work to focus on *Oprm1*, located at 6.75 Mb. At the time that this decision was made, global gene expression data had been collected using mPFC, NAc, and Vmb tissue from MADR mice. However, the more comprehensive network analyses using these data had not yet been completed (Belknap J. K. *et al.*, 2013). Examination of the initial dataset revealed that *Oprm1* was DE, with MALDR mice having greater expression in the mPFC, compared to MAHDR mice. Though other genes were also DE, focus was placed on *Oprm1* and the MOP-r system for three reasons: (1) because a MOR intake QTL that appeared to involve *Oprm1* had been mapped to the same region and it was considered that it could have common effects on drugs of abuse (Berrettini W. H. *et al.*, 1994a; Berrettini W. H. *et al.*, 1994b; Ferraro T. N. *et al.*, 2005); (2) there was an association of *OPRM1* with MA-induced-psychosis and dependence (Ide S. *et al.*, 2004a; Ide S. *et al.*, 2006; Dlugos A. M. *et al.*, 2011); and (3) there was evidence of MOP-r involvement in MA-related effects (Jones D. N. & Holtzman S. G., 1994; Lan K. C. *et al.*, 2008).

There were three main goals of this dissertation research project. The first goal was to examine potential differences in sensitivity to MOP-r mediated effects in MADR mice. Based on positive results, the second goal of this project was to investigate the efficacy of MOP-r drugs to alter MA intake and drinking patterns. These studies administered either MOP-r agonist or antagonist drugs in a limited access two-bottle choice MA drinking procedure. The final goal of this project were to verify the existence of the Chr 10 QTL for MA consumption using a more isogenic background and then to gain better mapping resolution of the Chr 10 QTL. This aim was addressed using congenic strains of mice, which were created from B6 and D2 inbred mouse strains, the progenitor strains of the selected lines.

**Table 6.1: Summary of differences in MOP-r agonist drug sensitivity**

| Phenotype | Results |
|---|---|
| Hot plate | MADR mice did not differ in latency to withdraw hind limb from hot plate following pretreatment with MOP-r agonist, fentanyl.<br><br>MADR mice did not differ in latency to withdraw hind limb from hot plate following pretreatment with MOP-r agonist, morphine. |
| Tail Flick | MADR mice did not differ in latency to withdraw tail from water bath following pretreatment with MOP-r agonist, fentanyl.<br><br>MADR mice did not differ in latency to withdraw tail from water bath following pretreatment with MOP-r agonist, morphine. |
| Magnesium sulfate abdominal writhing | MADR mice did not differ in the number of magnesium sulfate-induced abdominal writhes following pretreatment with MOP-r agonist drug, fentanyl. |
| Locomotor stimulation | MALDR-2 mice had greater locomotor stimulation to MOP-r agonist drug, fentanyl, compared to MAHDR-2 mice.<br><br>MALDR-2 mice had greater locomotor stimulation to MOP-r agonist drug, morphine, compared to MAHDR-2 mice. |
| Two-bottle choice drinking | MALDR-2 mice consume more MOP-r agonist, morphine, in a morphine vs. quinine two-bottle choice drinking procedure, compared to MAHDR-2 mice.<br><br>MALDR-2 mice consumed more MOP-r agonist, morphine, in a saccharin fading morphine vs. water two bottle choice procedure, compared to MAHDR-2 mice |

***Differences in sensitivity to and avidity for MOP-r agonist drugs***

Chapter 2 of this dissertation addressed the first thesis goal. I predicted that if line differences were seen, they would correspond with differences between the D2 and B6 progenitor strains, because the Chr 10 QTL accounts for > 50% of the genetic variance in MA intake, and a high frequency of D2 alleles in this region was associated with higher MA intake. Thus, I predicted that the MAHDR line would resemble the D2 strain and the MALDR line would resemble the B6 strain; in fact, D2 mice consume greater amounts of MA compared to B6 mice (Eastwood E. C. & Phillips T. J., 2012). MADR line differences on the nociception tests were not detected, indicating that sensitivity to the analgesic effects of MOP-r drugs is not genetically correlated with MA drinking, and that differences of the progenitor strains in analgesic response to MOR (Mogil J. S. *et al.*, 1999b) are not related to their difference in MA intake.

As shown in Chapter 2, the MADR lines did differ in sensitivity to the locomotor stimulant effects of MOR and FENT, with greater stimulation in MALDR than MAHDR mice (Eastwood E. C. & Phillips T. J., 2012). The psychomotor stimulant theory of addiction posits that the same DA mechanism influencing locomotor response also influences positive reinforcement (Wise R. A. & Bozarth M. A., 1987). Low doses of MOR (3 mg/kg) increased locomotor activity in B6, compared to D2, mice during a 3 h session, which positively correlated with DA efflux in the ventral striatum (Murphy N. P. *et al.*, 2001). However, if there was a direct relationship, then it would be expected that the B6 strain, which has greater locomotor stimulation to MOR (Phillips T. J. *et al.*, 1994b; ZioLkowska B. *et al.*, 2015), compared to the D2 strain, should also have greater sensitivity to MOR-induced reward. In fact, B6 and D2 mice both showed opioid-induced CPP, and the magnitude of CPP was actually greater in D2 mice, compared to B6 mice (Cunningham C. L. & Noble D., 1992). On the other hand, the MALDR line had greater acute locomotor stimulation to the MOP-r agonist drugs, MOR and FENT, compared to MAHDR line mice and also showed greater avidity for MOR-containing solutions (further discussed below). MAHDR mice exhibit greater stimulation to some doses of MA (unpublished), but not all

(Shabani S. *et al.*, 2011), and show greater MA-induced CPP (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011), supporting a partial relationship in these lines.

In Chapter 4, I examined voluntary oral preference for MOR in MADR mice. I predicted that B6 alleles in the Chr 10 QTL region would be associated with higher MOR intake, consistent with previous data (Berrettini W. H. *et al.*, 1994a; Berrettini W. H. *et al.*, 1994b; Ferraro T. N. *et al.*, 2005), and thus, that MALDR mice would consume more MOR than MAHDR mice. Two different two-bottle choice drinking procedures were used. One compared MOR-saccharin vs. quinine-saccharin intake, and the other used a MOR-saccharin fading procedure to ultimately compare MOR vs water on the final 4 d of the procedure (Belknap J. K. *et al.*, 1993c; Berrettini W. H. *et al.*, 1994b). Examination of taste preferences in the MADR mice demonstrated that MADR mice do not differ in saccharin or quinine consumption, so we did not anticipate the quinine vs saccharin procedure to be biased toward one line (Wheeler J. M. *et al.*, 2009; Shabani S. *et al.*, 2011). The saccharin fading procedure used by the Berrettini lab was used to map the MOR consumption QTL on murine Chr 10, in the same region as our identified QTL for MA intake (Berrettini W. H. *et al.*, 1994a). In both MOR drinking procedures, the MALDR line consumed more MOR compared to the MAHDR line. These data suggest that a negative genetic correlation exists between MA intake and MOR intake.

Combined, our inbred strain MA drinking data, MOR and FENT locomotor stimulation data, and MOR drinking data indicate that the MAHDR line resembles the D2 inbred strain, whereas the MALDR line resembles the B6 strain, for these traits. The data presented here suggest that acute locomotor sensitivity to MOP-r agonist drugs is influenced by genetic factors relevant to MA avidity, such that greater avidity is associated with reduced MOP-r agonist sensitivity. These data are summarized in Table 6.1. However, there are alternative interpretations. These studies utilized only one set of the replicate MADR lines and it is possible that the correlated responses for MOP-r related traits with MA intake are spurious. Spurious correlations, in the context of selected lines, are false-positive relationships (i.e., not true genetic

correlations) between a new measure and the selected trait (Henderson N. D., 1989b, 1997). Genetic drift, resulting in inbreeding at irrelevant loci in one line and not the other, may influence the behavioral differences observed between selectively bred lines. At the time these experiments were performed, the first set of replicate lines had already been retired, so data were collected only in replicate 2. Currently, a third replicate set of MADR lines exists and a fourth replicate set is under development. A future direction should be to confirm some of the findings reported here. I have already confirmed the genetic correlation for FENT-induced locomotor activation in the third replicate set of MADR lines (Eastwood, unpublished data), providing support for a genetic correlation between sensitivity to FENT-induced activation and MA drinking. However, although this could be due to pleiotropic gene effects, another possibility is the effect of a linked gene.

Based on alleles being in disequilibrium in a finite breeding population, such as in the selected lines where non-random mating occurs, it is likely that a haplotype block exists in the Chr 10 QTL region for MA intake. Haplotype blocks are collections of specific alleles in clusters of tightly linked genes on a Chr that are inherited together (Mackay T. F., 2001b). A Chr 10 haplotype block might have resulted in a QTL for MOR sensitivity being inherited with the QTL for MA intake in the MADR mice, such that differences in MOR sensitivity are not in fact, due to the pleiotropic effect of one or more genes that influence MA intake. This potential presence of a haplotype block that includes *Oprm1*, allelic variants of which influence MOP-r sensitivity and other MOP-r-related traits, should be considered in the interpretation of the current data.

Genotyping performed on Chr 10 in the second (Belknap J. K. *et al.*, 2013) and third replicate sets of MADR mice (Phillips, unpublished) confirms the QTL and a greater frequency of D2 alleles in this region in MAHDR mice, compared to MALDR mice. Thus, the same results for sensitivity to MOP-r drugs could be found in these replicates, whether due to pleiotropism or linkage. To sort this out, these linkages would need to be broken up.

**Table 6.2: Summary of the opioid pharmacokinetic and binding data from MADR, B6 and D2 mice**

| Phenotype | Results |
|---|---|
| [$^3$H]DAMGO binding (Bmax and $K_D$) | **Bmax:**<br>mPFC: MALDR>MAHDR<br>NAc: MALDR=MAHDR<br>Vmb: MALDR=MAHDR<br>**$K_D$:**<br>mPFC: MALDR=MAHDR<br>NAc: MALDR=MAHDR<br>Vmb: MALDR=MAHDR |
| MOR clearance (peak blood level and clearance) | MALDR=MAHDR for peak blood levels and drug clearance. |
| FENT clearance (peak blood level and clearance) | MALDR=MAHDR for peak blood levels and drug clearance. |

### *Opioid pharmacokinetics in MADR mice*

A summary of the pharmacokinetic and binding data is shown in Table 6.2. To determine whether the observed differences in sensitivity to MOP-r agonist-induced locomotor stimulation in the MADR lines were potentially related to differences in drug clearance patterns, peak blood levels and levels at several time points after acute MOR or FENT administration were determined. No significant differences were found between the MADR selected lines. One limitation of this analysis is that blood levels were assessed rather than brain levels. It is possible that different amounts of the opioid reached brain targets critical to the stimulant response. One assessment of whole brain concentration (minus the cerebellum) of MOR detected higher brain concentrations in B6 mice, compared to D2 mice (Belknap J. K. *et al.*, 1989), and this is the strain that shows the greater stimulant response. It is possible that analysis of brain concentrations rather than blood levels would have detected line differences that match those observed in the inbred strains. Further, because much of our focus in the MADR lines has been on particular brain regions used to examine gene expression, it is possible that assessing drug concentration in particular brain regions might have also produced different results.

#### MOP-r binding in MADR, B6 and D2 mice

[$^{3}$H]DAMGO MOP-r binding in the mPFC, NAc, and Vmb determined that the MALDR line had greater Bmax or apparent receptor number in the mPFC tissue compared to the MAHDR line, but the MADR lines did not differ in MOP-r density or affinity in the NAc or Vmb regions. These data are consistent with greater *Oprm1* expression in MALDR mice (Belknap J. K. *et al.*, 2013) being associated with greater protein production in the mPFC. I found no differences between B6 and D2 mice. However, another study examining the effects of either chronic saline or heroin treatment on MOP-r density and affinity, found that although saline-treated B6 and D2 mice did not differ for a number of brain regions, B6 mice had greater MOP-r density in the central lateral thalamic nuclei and the central medial thalamic nuclei compared to

D2 mice. B6 mice also had greater DAMGO stimulated [$^{35}$S]GTPyS binding, compared to D2 mice in both the NAc core and shell (Bailey A. *et al.*, 2010). Also in contrast to the data shown here, a previous study found that the D2 inbred strain had greater MOP-r Bmax and brain concentration of a radiolabeled $^{14}$C-MOR compound 30 min after administration, compared to B6 mice (Belknap J. K. *et al.*, 1989). However, those data were collected in whole brain tissue (minus the cerebellum) and used [$^{3}$H]naloxone as the radioligand. Additionally, the use of a different radioligand with a different specificity for the MOP-r could have conferred differences in the sensitivity of the assay.

**Table 6.3: Summary of MA microstructure and corresponding blood MA levels in MADR mice.**

| Phenotype | Results |
|---|---|
| MADR in a lickometer device | MADR mice consumed similar amounts of MA on D1 of MA access but MAHDR mice took more licks from the MA-containing bottle on subsequent days.<br># of bouts: MAHDR>MALDR<br>Bout length: MAHDR>MALDR<br>Interbout interval: MAHDR<MALDR<br>Latency to first bout: MAHDR>MALDR |
| MA blood concentration | D1 (20 mg/l) 4h: MAHDR = MALDR<br>D4 (20 mg/l) 2h: MAHDR > MALDR<br>4h: MAHDR > MALDR<br>D8 (40 mg/l) 2h: MAHDR > MALDR<br>4h: MAHDR > MALDR<br>D12 (80 mg/l) 2h: MAHDR > MALDR<br>4h: MAHDR > MALDR |

**MA drinking microstructure and blood levels in MADR mice**

To assess MA consumption patterns in the MADR lines before progressing to the second major aim of this project, patterns of MA intake were examined during 4-h limited access sessions. Microstructural analysis of MA intake was completed in drug-naïve animals to determine temporal patterns of MA drinking, before a subsequent experiment was performed that examined the effect of FENT on MA intake. In addition, blood MA levels were examined during MA drinking. An earlier study used an operant procedure, in which MADR mice were trained to lever press to gain access to a MA-containing tube (Shabani S. et al., 2012a). MAHDR and MALDR mice did not differ in MA intake on the first day of MA access, but on each subsequent day the MAHDR line consumed more MA than the MALDR line. Similar results were obtained in the current lickometer study, in which MA intake and MA blood levels did not significantly differ between MALDR and MAHDR mice on the first day of MA access. After this time, the line difference in MA intake emerged and significant correlations were found between MA intake and MA blood levels. MAHDR mice consumed relatively constant amounts of MA throughout the drinking session, and consumed significantly more MA when higher concentrations of MA were offered. In a previoius study, a difference in peak MA blood levels was found 15 min after an acute 2 mg/kg MA treatment, with MAHDR mice showing a higher MA level than MALDR mice; however, the lines did not differ in rate of MA clearance. Thus, differences in MA intake do not appear to be due to differences in the rate or ability to metabolize MA (Shabani S. *et al.*, 2012b). In the current study, blood MA levels were examined following voluntary oral intake. It is unknown whether mice titrated their intake to maintain a certain blood (or brain) level of MA, but our data confirm that the MAHDR line consumes biologically relevant amounts of MA that correspond with blood MA levels. A summary of these data is included in Table 6.3. This information was used to design later experiments that examined the effects of pharmacological agents on MA intake during the first 4 h of the dark in the light:dark cycle.

**Table 6.4: Summary of the effects of MOP-r drugs on MA intake in MADR mice**

| Phenotype | Results |
|---|---|
| Morphine pretreatment on MA intake | Morphine pretreatment (5, 10, or 15 mg/kg) dose-dependently reduced MA intake in MAHDR-2 mice but not in MALDR-2 mice. However, total volume consumed was dose-dependently reduced in both MADR lines. |
| Buprenorphine pretreatment on MA intake | Low doses of Buprenorphine (1 or 2 mg/kg) reduced MA intake in the MAHDR-2 line during the first 4h of a 6-h two-bottle choice limited access drinking in the dark procedure. The 4 mg/kg dose did not significantly alter MA intake in the MAHDR-2 mice. Buprenorphine treatment did not alter MA intake in the MALDR-2 mice. |
| Fentanyl pretreatment on MA intake in a lickometer device | FENT pretreatment (0, 0.1, or 0.2 mg/kg) did not alter MA intake or total volume consumed in the 4 h session, but reduced licks from the MA-containing bottle during the first 30 min of the drinking session. |
| Naltrexone pretreatment on MA intake | Naltrexone pretreatment (0.5, 1, 2, 5, 10, or 20 mg/kg) did not alter MA intake or total volume in either selected line in a 6-h limited access two-bottle choice drinking in the dark procedure. |

***Pharmacological manipulation of MA drinking***

The second goal of this dissertation project was to examine whether MOP-r agonist drugs could reduce MA intake in the MAHDR line. It was hypothesized that the MAHDR line would more closely resemble MALDR mice for MA intake, when given a MOP-r agonist prior to MA drinking. With regard to neurochemical mechanisms, more MOP-rs in the mPFC of the MALDR line could result in a greater reduction in levels of GLU neuron firing when MA is ingested compared to the MAHDR line. This would be expected to reduce DA levels in MALDR mice, and potentially enhance the aversive effects of MA or decrease sensitivity to its rewarding effects.

The ability of MOP-r agonist and antagonist drugs to alter MA intake was assessed in several studies using a limited-access drinking in the dark, two-bottle choice drinking procedure. These studies were designed, in part, using information gained from ethanol drinking-in-the-dark two-bottle choice procedures and operant ethanol self-administration studies (Altshuler H. L. *et al.*, 1980; Phillips T. J. *et al.*, 1997; Kamdar N. K. *et al.*, 2007). My studies included either a 4- or 6-h limited access drinking session, when mice were offered a water and MA-containing tube as soon as the lights turned off. Though our experimental design did not initiate the drinking session 3 h into the dark cycle, as is typical of the ethanol drinking-in-the-dark procedure, some of our data were collected for 6 total h, 3 of which occurred 3 h into the dark cycle. The duration of these experiments was based on the pharmacological activity of the MOP-r agonist and antagonist drugs used in the drinking procedures. Drinking procedures that used the lickometer apparatus collected data for a total of 4 h, which were hours 0-4 of the 6-h procedure. This experimental design allowed the effects of FENT on MA intake to be examined with greater temporal resolution. Regardless of MOP-r drug pretreatment or duration of the drinking procedure, most of the opioid drug-dependent effects on MA intake occurred during the first 2 h of the 6-h drinking session, and were specific to the MAHDR line only. In addition, the MA vs water lickometer drinking study, discussed in Chapter 3, which recorded cumulative patterns of

drinking over a 4 h drinking in the dark procedure, demonstrated that MADR mice consistently took licks from both the MA- and water-containing tubes throughout the drinking session. Though the MAHDR line took more licks from the MA-containing bottle compared to the MALDR, when intake was examined in 30-min time periods, MA intake was consistent across the 4-h session for each line. These data suggest that initiating the drinking session earlier than 3 h into the dark cycle was unlikely to have affected drinking behavior in MADR mice during the limited access drinking procedures. Thus, it does not appear that the procedural differences between our limited access drinking procedures and those used by others have impacted the reported data.

In Chapter 4, I demonstrated that lower doses of the partial MOP-r agonist drug, BUP, reduced MA intake during the first 4 h of a two-bottle choice 6-h drinking session. A U-shaped dose-response pattern of BUP effect on MA intake was interpreted to indicate partial agonist activity at lower doses and antagonist activity at higher doses. That naltrexone did not alter MA intake in MAHDR mice (Eastwood E. C. & Phillips T. J., 2014) is consistent with the lack of effect of BUP at higher doses that have antagonist effects. Other receptors may also have been involved in the effects of BUP. It is possible that BUP acted at kappa or delta opioid receptors (Richards M. L. & Sadee W., 1985; Leander J. D., 1987; Negus S. S. *et al.*, 2002). Low doses of BUP (0.03 and 0.3 mg/kg) increased ethanol intake, whereas high doses (3.0 and 6.0 mg/kg) decreased ethanol intake, and pretreatment with naltrexone prevented the BUP-induced increase in ethanol intake. Future investigations into BUP effects on MA intake should focus on a lower dose range, and the associated mechanisms.

The effects of selective MOP-r agonist drugs were examined in Chapter 5. MOR dose-dependently reduced MA intake during the first 4 h of the 6-h session, when the lower MA concentration was offered, but did not reduce MA intake when the higher concentration of MA was offered. MOR also dose-dependently reduced general fluid intake and reduced the consumption of a preferred natural reward, saccharin. These data suggest that MOR induces a

behavior that competes with the ability to perform drinking behavior, rather than affecting a specific mechanism associated with MA intake. However, examination of total volume consumed in the MOR-MA and MOR-saccharin studies revealed that roughly 125% greater total volume was consumed when the 0.33% and 0.66% saccharin concentrations (126.8 ± 6.7 and 163.7 ± 9.4 ml/kg, respectively) were offered, compared to total volume consumed when 20 mg/l and 40 mg/l MA concentrations (78.8 ± 3.4 ml/kg and 97.9 ± 3.6 ml/kg, respectively) were offered. Further, the 15 mg/kg dose of MOR time-dependently reduced total volume consumed in both the MA vs water and saccharin vs water drinking procedures. In the MA vs water study, the 15 mg/kg dose of MOR reduced MA intake by approximately 85% when the 20 mg/l MA concentration was offered and by approximately 80% when the 40 mg/l MA concentration was offered. In the saccharin vs water study, the 15 mg/kg dose of MOR reduced total volume consumed by 50% when the 0.33% solution of saccharin was offered and by approximately 45% when the 0.66% solution of saccharin was offered. The reduced magnitude of effect of MOR on total volume in the saccharin study may indicate that saccharin is more highly preferred and intake is more difficult to disrupt. On the other hand, different patterns of drinking of saccharin vs MA would be expected, based on the pharmacological effects of MA that are absent for saccharin.

Based on MA drinking microstructure in MADR mice, predictions regarding potential changes in drinking pattern were made for the FENT lickometer study. Specifically, it was predicted that MOP-r agonist drugs would increase the latency to first MA bout, decrease the interbout interval, and decrease the number and duration of bouts in MAHDR mice. FENT has transient effects, with a short half-life (~4.9 min in brain tissue) (Kalvass J. C. & Pollack G. M., 2007). I implemented a lickometer procedure to potentially pick-up time-dependent effects that might not be seen in 2 hour volume readings. However, no apparent effects of FENT pretreatment on MA intake or drinking microstructure were found. A summary of the effects of MOP-r drugs on MA intake is presented in Table 6.4.

One limitation to the lickometer device is that licks are recorded every time that a rodent closes an open circuit between the sipper tube and wire floor by standing on the wire floor and making contact with a tube. This contact may occur from a rodent bumping into or playing with a tube rather than taking a lick from the tube. Though this procedure is validated by correlating volume consumed with the number of licks taken from the corresponding bottle, it is still possible that not every lick that is counted is a 'true' lick. The Biodaq is a novel apparatus that has the ability to assess microstructure of drinking based on changes in weight of the tubes offered to rodents, as well as food intake. Weight sensors in the apparatus record the weight of the fluid-containing tube every second and register whether eating or drinking has commenced when 0.08g or more of force is applied to the food hopper or tube. Bout information can also be extracted from the recorded data (see vendor website for additional information about the system and data collection method: http://researchdiets.com/biodaq/index/htm). The Biodaq system has been used previously to assess ethanol drinking microstructure (Barkley-Levenson A. M. & Crabbe J. C., 2012). Use of the Biodaq system may have resolved concerns of false licks, especially if false contacts were being reported that did not result in a change in volume from the tubes.

Though significant correlations were detected between MA intake and licks from the MA-containing tube, and also between water consumed and licks from the water-containing tube, the correlations were lower than those described in other published lickometer studies. Thus, some contacts may not have been licks. Across the two lickometer studies, correlations between 0.5-0.78 were generated; other published studies have reported correlations upwards of r=0.9 (Ford M. M. *et al.*, 2005; Ford M. M. *et al.*, 2009; Sharpe A. L. & Phillips T. J., 2009). However, those studies focused on manipulating ethanol drinking that had already been established. Sharpe & Phillips (2009) and Ford et al. (2009) both focused on home-cage ethanol drinking, rather than examining drinking behavior in a chamber that is separate from the home-cage. The correlations between intake and licks from the fluid-containing tube might have been

stronger in MADR mice had home-cage drinking been examined. Some of these experimental designs implemented criteria to establish stable ethanol drinking. In one case, mice had to have less than 10% variability in ethanol consumed over 3 consecutive drinking sessions before the pretreatment was administered (Ford M. M. *et al.*, 2005; Ford M. M. *et al.*, 2009). Those studies did not report the average amount of time it took for each mouse to reach criterion before progressing to the next phase of the experiment. It is likely that stronger correlations would have been obtained between the amount of fluid consumed and licks taken for the corresponding bottle had a longer acclimation period been possible. However, the goal of my experiments was to examine the effects of FENT on the acquisition of MA intake rather than the effects of FENT on established MA intake.

As mentioned, in Chapter 4, I also examined the effect of the MOP-r antagonist drug, naltrexone, on MA intake. Naltrexone attenuated the expression of MA-induced locomotor sensitization and suppressed the induction of MA-induced sensitization (Chiu et al., 2005). Naltrexone has also been shown to reduce craving for AMPH, as well as the subjective effects produced by AMPH in AMPH-dependent subjects (Jayaram-Lindstrom N. *et al.*, 2004; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b; Tiihonen J. *et al.*, 2012). Thus, although it was possible that naltrexone would reduce MA intake in MAHDR mice, I hypothesized that if MOP-r agonist drugs reduced MA intake in the MAHDR line, then perhaps the antagonist would enhance drinking in the MALDR mice. Naltrexone across a large range of doses (0.5-20 mg/kg) did not alter MA intake in either of the selected lines. Therefore, I obtained no evidence for MOP-r antagonism to either discourage or encourage MA intake.

One additional factor should be considered. Naltrexone therapy is commonly used to treat opioid and alcohol dependence and certain *OPRM1* polymorphisms have been associated with better treatment outcomes. Specifically, the A118G polymorphism in exon 1 of *OPRM1* causes an A to G amino acid change that results in the loss of an N-glycosylation site in an extracellular receptor region. In cell lines, this polymorphism causes less *OPRM1* mRNA to be

produced but nearly three-fold more β-endorphin binding is seen. Naltrexone therapy in alcohol-dependent subjects carrying the G allele had better treatment prognosis, with lower rate of relapse, and a longer time to return to heavy drinking, compared to subjects carrying the A allele (Setiawan E. *et al.*, 2012; Marini V. *et al.*, 2013). Though these studies have specifically examined the efficacy of naltrexone to reduce ethanol intake, it is possible that the A118G polymorphism may also influence the efficacy of MOP-r drugs to reduce MA use in humans with MA-dependence. However, when this was recently examined, no greater efficacy of naltrexone to alter days of abstinence or other markers of MA use in MA-dependent individuals with the A118G polymorphism was found (Pal R. *et al.*, 2015). There is some evidence that naltrexone attenuated the subjective effects of and craving for MA (Jayaram-Lindstrom N. *et al.*, 2005; Jayaram-Lindstrom N. *et al.*, 2008a; Jayaram-Lindstrom N. *et al.*, 2008b). However, in a more recent study naltrexone did not alter the physiological effects of AMPH (Comer et al., 2013). These findings support the data reported here, where no dose of naltrexone affected MA drinking. Furthermore, when given in combination with bupropion, a DAT and NET reuptake inhibitor, naltrexone did not reduce the reinforcing effects of intranasal MA (Stoops W. W. *et al.*, 2015). However, naltrexone in combination with alprazalom, a positive allosteric modulator of GABA, reduced more subjective effects of oral AMPH than did either drug alone (Marks K. R. *et al.*, 2014). These data suggest that in combination with certain other drugs, naltrexone may offer an effective treatment for MA and AMPH dependence, but that additional research is needed.

A previous *in vivo* microdialysis study (Lominac K. D. *et al.*, 2014) demonstrated that MAHDR mice had lower basal extracellular DA content in the NAc and mPFC, compared to MALDR mice. These findings in combination with my earlier predictions regarding DA, GABA, and GLU projections from the VTA, NAc, and mPFC are depicted in Fig. 6.1, panels A and C. Following an acute dose of MA, DA changes were measured and no line differences were detected in the NAc; however, MAHDR mice had increased extracellular DA levels in the PFC compared to MALDR mice (Fig. 6.1 B, D). These findings indicating that MAHDR mice

experience greater MA-induced activation of reward circuitry may explain the enhanced reward and reinforcement sensitivity of these mice. These results also suggest that my predictions regarding increases in DA following MA treatment (Fig. 1.1) were not entirely correct. In Fig. 1.1, I had generally predicted that MA would increase DA in both the NAc and PFC. However, at least in the genetically susceptible MAHDR mice, DA was increased only in the PFC after MA exposure. This is now represented in Fig. 6.1.

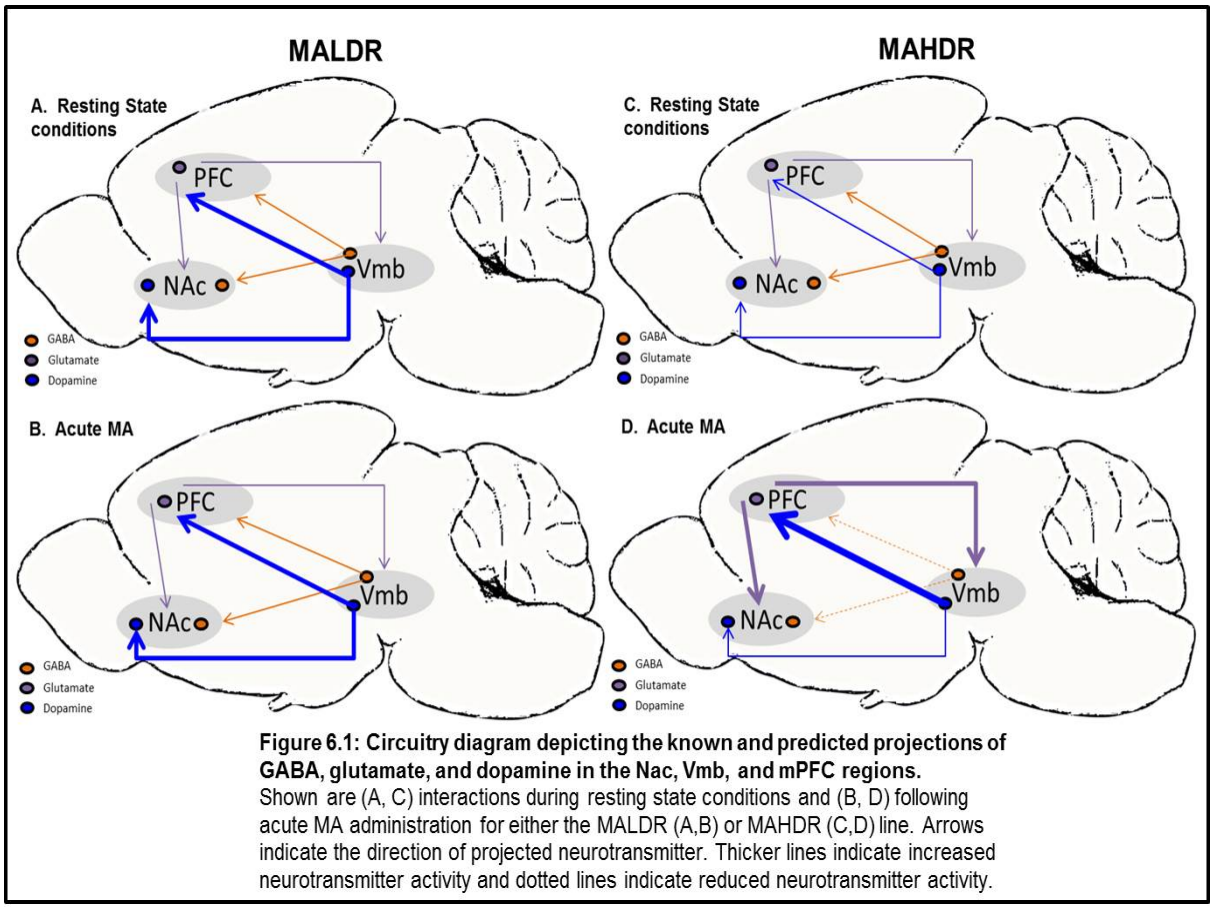


**Figure 6.1: Circuitry diagram depicting the known and predicted projections of GABA, glutamate, and dopamine in the Nac, Vmb, and mPFC regions.** Shown are (A, C) interactions during resting state conditions and (B, D) following acute MA administration for either the MALDR (A,B) or MAHDR (C,D) line. Arrows indicate the direction of projected neurotransmitter. Thicker lines indicate increased neurotransmitter activity and dotted lines indicate reduced neurotransmitter activity.

It is worth noting that the mPFC has two subdivisions, the infralimbic (IL) and prelimbic (PL) cortices, which are anatomically and functionally distinct. Lominac et al. (2014) did not focus on a particular region of the mPFC. The PL cortex has been associated with initiating conditioned responses and drug-seeking behaviors, while the IL has been associated with extinction of conditioned reward and drug-seeking behaviors (Gass J. T. & Chandler L. J., 2013). It is possible that had Lominac et al. (2014) examined the two subdivisions of the mPFC

(IL vs. PL) that the PL subdivision would have been identified as the region where increased extracellular levels of DA occur in response to MA. In the current binding assays and previously discussed global gene expression analyses (discussed in Chapter 5), the PL subdivision of the mPFC was isolated. This region seemed most appropriate to focus on because of its association with other psychostimulant drug effects (McFarland K. & Kalivas P. W., 2001; McFarland K. *et al.*, 2004).

**Verification and Finer Mapping of a QTL for MA intake**

The involvement of *Oprm1*, which is in the Chr 10 QTL region for MA drinking, was further investigated using Chr 10 D2.B6 congenic mice (Chapter 5). As discussed in Chapter 2, the MAHDR line resembles the D2 strain and the MALDR line resembles the B6 strain, with regard to some, but not all, MA- and opioid-related traits. Finer mapping of a Chr 10 QTL for MOR intake using D2.B6 congenics mapped the QTL from a 28.8 Mb to a 9.5 Mb region that included *Oprm1* (Ferraro T. N. *et al.*, 2005; Doyle G. A. *et al.*, 2008). However, congenic mapping for MA intake excluded the Chr 10 0-7.58 Mb interval (the region between 7.58 and 7.72 Mb is of unknown origin, so cannot be ruled out), and thus, excluded *Oprm1*, which resides at 6.75 Mb. The current analysis, using just 2 congenic strains, reduced the Chr 10 QTL interval from 40 to 12.86 Mb. DE of specific genes can be used to narrow focus on particular candidates, because likely to have a functional impact and specific However, other genes cannot be ruled out and there are 55 known protein-coding genes in this interval. It is also possible that a gene(s) within the Chr 10 QTL interval that is not DE contains a SNP that alters response to MA, for example, via reduced or lost function in the absence of changed expression. Therefore, finer mapping is needed to reduce the number of genes for further consideration.

In total, the Affymetrix microarray gene expression analysis identified 231 genes residing on Chr 10 that were DE between MADR mice in mPFC, NAc, or Vmb tissue. Of these genes, 93 were located in the initially mapped 40 Mb Chr 10 QTL interval (Belknap et al., 2013). However,

the Chr 10 QTL accounts for ~50% of the genetic variance in MA intake, so other genes in other regions must also have an impact. However, some potential genes of interest in the Chr 10 QTL region include neuromedin B, *Nmbr*, at 14.4 Mb, G-protein receptor 126, *Gpr126*, at 14.09 Mb, human immunodeficiency virus type 1 enhancer binding protein, *Hivep2*, at 13.8 Mb, interferon gamma receptor 1, *Ifngr1*, at 19.28 Mb, peroxisome biogenesis factor 7, *Pex7*, at 13.2 Mb, and phosphodiesterase 7B, *Pde7b*, at 20.08 Mb. *Pde7b* has been linked to brain neurodegeneration as well as inflammation, and silencing its activity in DA neurons conferred a neuroprotective role in a mouse model of Parkinson's disease (Morales-Garcia J. A. *et al.*, 2015). There are data to suggest a partial overlap between MA-induced striatal damage and neurodegeneration in Parkinson's disease; thus, this could be an interesting candidate to investigate. Data suggesting a role for neuroinflammatory genes are consistent with previously published data in the selected lines, where MA-induced gene expression differences in NAc tissue, were measured in the first replicate MADR mouse lines using a qPCR array. The Gene Ontology database, NIH DAVID, was used to identify functional characteristics of DE genes after MA exposure of MAHDR and MALDR mice and found gene pathways associated with apoptosis, inflammation and cell survival (Wheeler et al., 2009). *Nmbr* is expressed in the dorsal horn of the spinal cord, and plays a role in the perception of thermal pain, regulating body temperature and stress responses (Ohki-Hamazaki H. *et al.*, 1999; Mishra S. K. *et al.*, 2012). MA induces changes in body temperature and *Nmbr* could be involved in the differences between MAHDR and MALDR mice in sensitivity to MA-induced hypothermia or hyperthermia (Harkness et al., 2015).

One approach to finer map is to utilize the Chr 10 D2.B6 0-20.4 Mb congenic strain to create additional interval specific congenic strains, in which the donor segment is broken up into smaller intervals. Other strategies to finer map the QTL interval could include selective phenotyping, recombinant progeny testing, or the recombinant inbred segregation test (RIST). During selective phenotyping, a large F2 population is produced (~1500 for dominant traits) and

based on genotyping results, only individuals (~300) that are recombinant in the already determined QTL interval are phenotyped (Darvasi A., 1998). Recombinant progeny testing relies on crossing by breeding one individual that is recombinant on a chromosome in the region of interest with one of the progenitor inbred strains. The resulting offspring can be genotyped to determine if the recombinations in the QTL region were passed on. This approach requires large samples and is ideal for QTLs with large and dominant effects and requires producing and phenotyping roughly 400 animals (Darvasi A., 1998, 2001; Liu X. *et al.*, 2001). Our QTL has a large effect size, so this approach could be successful. The RIST utilizes already existing RI strains that contain recombinations in the region of the detected QTL. To create the RIST population, one RI strain is crossed with one of the progenitor strains (P1) to form an F1 population, which is then intercrossed by breeding with the other progenitor strain (P2) to form a backcross population (BC1). This is reciprocally performed to form a second backcrossed population (BC2). The two backcross populations are genotyped for several markers and then the BC1 population is genotyped for markers that reside in the region where the P2 alleles are located in the RI strain. Since the QTL has been previously mapped to this region, the QTL will segregate in one of the two BC populations but not the other. The two BC populations are then phenotyped and the population that the QTL segregated in can be determined and the QTL location can be resolved based on the location of the recombination point. This strategy only requires 2 generations of offspring and approximately 200 individuals to reduce the QTL interval from 25 cM to 5 cM (Nissenbaum J. *et al.*, 2010).

Finally, MA intake in the selected lines is a complex genetic trait influenced by multiple genes. The identified Chr 10 QTL accounted for approximately 50% of the genetic variance in MA consumption. The QTL analysis did identify significant QTLs at other locations, specifically on Chr 2, 9 and X (Belknap et al., 2013). Furthermore, rather than a single gene, a cluster of genes on Chr 10 could be responsible for the large amount of genetic variance accounted for at this location. The cluster of DE genes in the Chr 10 QTL region could support this hypothesis,

although it is also possible that a single gene has a wide impact on the expression of multiple genes. For example, DE genes that were identified between the MADR lines, which were not associated with a detected QTL, could be trans-regulated by a gene within a QTL region.

**Summary and Conclusions:**

The findings presented in this dissertation provide evidence that *Oprm1* is likely not a QTG influencing MA intake on Chr 10. Differences between the MADR lines in sensitivity to MOP-r agonists and in MOR intake may be most parsimoniously explained as the effect of linkage. However, although *Oprm1* may not be a QTG for MA intake, a role for *Oprm1* regulation and MOP-r-mediated opioid pathways cannot be completely ruled out. Some doses of MOP-r agonist drugs were found to reduce MA intake in the MAHDR line. However, a more specific effect of a MOP-r partial agonist was found than of more selective MOP-r agonists, and could indicate an alternative mechanism of action. Although these data provide some evidence that MOP-r agonist drugs may offer some efficacy in reducing MA intake, additional data are needed. In addition, when added to a risk network for MA intake, *Oprm1* was identified as a significant hub, that was regulated by multiple genes in that network (Belknap et al., 2013). Thus, it may be a downstream target, rather than playing a role in risk for MA use.

**Future Directions:**

One primary future direction should be finer mapping of the Chr 10 QTL. *Oprm1* was eliminated from further consideration as a QTG on Chr 10 for MA drinking, but many genes remain in the mapped region and examining 50-some genes, one at a time, is not efficient. An exception may be for genes for which a SNP has a profound effect on function. For example, a Chr 10 gene currently being examined is trace amine-associated receptor 1 (*Taar1*). MA and other AMPH-like psychostimulants serve as agonists of the receptor (TAAR1) expressed by *Taar1*. *Taar1* knockout mice demonstrated enhanced MA-induced locomotor activity and MA-induced conditioned place preference (CPP), compared to wild type mice (Achat-Mendes C. *et al.*, 2012). Recent collaborative work in the Phillips and Janowsky labs, determined that the D2

strain harbors a non-functional *Taar1* allele that is unique to this strain (Harkness J. H. et al., 2015). Furthermore, they found that the non-functional allele segregates in the MAHDR line. Examination of MA intake in *Taar1* knockout mice showed high MA intake levels comparable to the MAHDR line, similar to high MA intake levels in D2 mice (Eastwood and Phillips, 2012), which can be considered a naturally occurring null mutant. These data suggest that MA consumption is regulated, in part, by *Taar1* (Harkness J. H. *et al.*, 2015).

Another direction could be to follow up the finding that *Oprm1* is regulated by genes in an important risk network identified by the analysis of DE genes in the MADR lines (Belknap J. K. *et al.*, 2013). MOP-r binding assays in MADR line mice were examined in only 3 brain regions. It is possible that differences important to MA drinking exist in other regions. However, it is also important to further examine the low doses effects of BUP that reduced MA drinking in the MAHDR line. Though other MOP-r drugs had effects, BUP produced the most profound effects on MA intake and that were not accompanied by overall reductions in fluid intake. To determine whether the effects of BUP were mediated through MOP-rs, additional experiments examining the ability of naltrexone to block the BUP-induced decrease in MA intake could be performed. Finally, the effects of opioid treatments on established MA drinking, should be considered. My studies concentrated on the potential role of opioid pathways in the development of MA intake in a genetic animal model of heightened risk for intake. However, the heightened MA intake in the MAHDR lines also makes them useful in medications development studies.

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