TRACE AMINE-ASSOCIATED RECEPTOR 1 REGULATION OF METHAMPHETAMINE INTAKE AND AVERSION

By

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LIST OF ABBREVIATIONS

+/+ – wildtype genotype

+/- – heterozygous genotype

-/- – knockout genotype

5-HT – serotonin

5-HT1A – serotonin receptor 1A

5-HT1B – serotonin receptor 1B

ACTH – adrenocorticotropin hormone

ANOVA – analysis of variance

Ar – androgen receptor gene

B6 – C57BL6/J

B6D2F2 – F2 generation of C57BL/6J and DBA/2J cross

BCA – bicinchoninic acid

bp – base pair

BXD – C57BL/6J and DBA/2J recombinant inbred line(s)

cAMP –3'-5'-cyclic adenosine monophosphate

Casp8 – caspase 8, apoptosis-related cysteine peptidase gene

CeA – central amygdala

cM – centimorgan

Cmax – maximum (peak) plasma concentration

CNS – central nervous system

CORT – corticosterone

CPA – conditioned place aversion

CPP – conditioned place preference

CRF – corticotropin-releasing factor

CS+ – conditioned positive stimulus

CS- – conditioned negative stimulus

CTA - conditioned taste aversion

D_1 – receptors

D_2 – receptors

D_3 – receptors

D2 – DBA/2J

DA – dopamine

DAT – dopamine transporter

***d*-isomer** – dextrorotatory isomer

dl – deciliter

DMSO – dimethyl sulfoxide

EC_{50} – half maximal effective concentration

EIA – enzyme Immunoassay

ELISA – enzyme-linked immunosorbent assay

ES – embryonic stem

***Esr1* –** estrogen receptor 1 gene

***Esr2* –** estrogen receptor 2 gene

EtOH – ethanol

EPPTB – N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide

eQTL – expression quantitative trait locus

F – female

F2 – second filial generation offspring

FCS – fetal calf serum

FLUX – fluoxetine

Fos – FOS gene

GABA – *γ*-aminobutyric acid

GFP – green fluorescent protein

GLU – glutamate

***Grm4* –** metabotropic glutamate receptor 4 gene

H^2 – heritability

HIV – human immunodeficiency virus

***Hivep2* –** human immunodeficiency virus type 1 enhancer binding protein 2 gene

h – hour

HMACT - high methamphetamine activation

HPA – hypothalamic-pituitary-adrenal axis

Hsce70 – immunoglobulin heavy chain binding protein 70 gene

Hsp40 – heat shock protein 40 gene

Htr3 – serotonin receptor 3 gene

Il6 – interleukin 6 gene

i.p. – intraperitoneal

i.v. – intravenous

KCl – potassium chloride

kg – kilogram

KO – knockout

l – liter

LC – locus coeruleus

LiCl – lithium chloride

***l*-isomer** – levorotatory isomer

LMACT – low methamphetamine activation

LOD – log of the odds

M – male

MA – methamphetamine

MADR – methamphetamine drinking mouse lines

MAHDR – methamphetamine high drinking mouse line

MAHSENS – methamphetamine high sensitization mouse line

MALDR – methamphetamine low drinking mouse line

MALSENS – methamphetamine low sensitization mouse line

***Map3k5* –** mitogen-activated protein kinase kinase kinase 5 gene

Mapk3 – ERK-1 MAP kinase gene

Mb – mega base

MDMA – 3,4-methylenedioxy-methamphetamine

µg – microgram

mg – milligram

mg/kg – milligram per kilogram

mGlur1 – metabotropic glutamate receptor 1 gene

Mpdz – multiple PDZ domain protein gene

mPFC – medial prefrontal cortex

NAcc – nucleus accumbens

NaCl – sodium chloride

NE - norepinephrine

NET – norepinephrine transporter

NIDA – National Institute on Drug Abuse

NISX – nisoxetine

nM – nanomolar

NFκB – nuclear factor of kappa light polypeptide

Nfkb1 – nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 gene

Nfkb2 – nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 gene

NTS – nucleus of the solitary tract

Oprm1 – μ opioid receptor gene

PCR – polymerase chain reaction

PFC – prefrontal cortex

pg – picogram

PKA – protein kinase A

PKC – protein kinase C

POMC – proopiomelanocortin

PVN –paraventricular nucleus of the hypothalamus

qPCR – quantitative polymerase chain reaction

QTG – quantitative trait gene

QTL – quantitative trait locus

Rela – NFκB subunit p65 gene

Rep – replicate

RI – recombinant inbred

RIPA – radioimmunoprecipitation assay buffer

SA – self-administration

SEM – standard error of the mean

SERT – serotonin transporter

Slc6a2 – NET gene

Slc6a4 – SERT gene

SNP – single nucleotide polymorphism

T – circadian free-running period

T_0AM – thyronamine

T_1AM – 3-iodothyronamine

Taar1 – gene encoding trace amine-associated receptor 1

TAAR1 – trace amine-associated receptor 1

UNODC – United Nations Office on Drugs and Crime

US – unconditioned stimulus

VAPORHCS – Veterans Affairs Portland Health Care System

VMAT2 – vesicular monoamine transporter 2

VMB – ventral midbrain

WHO – World Health Organization

WT – wildtype

UCP – uncoupling protein

UCP1 – uncoupling protein 1

UCP3 – uncoupling protein

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ABSTRACT

Continued methamphetamine (MA) use is dependent on a positive MA experience and is likely preempted by sensitivity to the aversive effects of MA. The bidirectional selective breeding of mice for high (MAHDR) and low (MALDR) voluntary MA consumption has consistently demonstrated an inverse genetic relationship between MA consumption and MA-induced conditioned taste aversion (CTA). The progenitors of the selected lines were individuals from the reciprocal F2 intercross of the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains. A quantitative trait locus (QTL) previously identified on mouse chromosome 10 in the selected lines accounts for greater than 50% of the genetically-determined difference in MA intake in the MA drinking (MADR) lines. The trace amine-associated receptor 1 gene (*Taar1*) is within the confidence interval of the QTL and encodes a receptor (TAAR1) for which MA is an agonist. Accumulating evidence suggests that *Taar1* is a quantitative trait gene (QTG) for MA intake. The D2 progenitor strain has a polymorphism in *Taar1*, resulting in loss of function of TAAR1.

The overarching goal of this dissertation was to further explore the relationships between sensitivity to aversive effects of MA and MA consumption. The first aim focused on the role of *Taar1* in MA consumption and sensitivity to MA-induced CTA. I found that MA drinking and sensitivity to MA-induced CTA corresponded with *Taar1* genotype. The segregation of the B6 *Taar1* allele in MALDR mice confirms the direction of allele influence predicted by the chromosome 10 QTL, and was dominant in its protective effect against MA

intake. Mice homozygous for the non-functional D2 *Taar1* allele showed significantly increased consumption over the heterozygous or B6 homozygous animals.

The second aim focused on physiological responses to MA that may impart greater sensitivity to MA-induced aversion. I hypothesized that high aversion and low intake would be genetically associated with greater sensitivity to the effects of MA on body temperature and on stress response. Additionally, I hypothesized that increasing stress axis activity while MA intake was being established would reduce MA intake in MAHDR mice. Increased hypothermic response and reduced hyperthermic response, as well as elevation of plasma corticosterone (CORT) following MA, corresponded with high sensitivity to MA-induced aversion and functional TAAR1. Furthermore, consumption of CORT in solution with MA decreased preference for MA in MAHDR mice. Therefore, hypothermia and elevated CORT response may be aversive effects of MA that contribute to limiting MA consumption.

The third aim of this dissertation was to investigate norepinephrine transporter (NET) and serotonin transporter (SERT) involvement in regulation of MA aversion. MAHDR mice have higher expression of nucleus accumbens (NAcc) NET and SERT than MALDR mice. I hypothesized that MALDR mice would be more sensitive to NET and SERT blockade-induced CTA. Additionally, I hypothesized that repeated blockade of NET or SERT would reduce sensitivity to MA-induced CTA through transporter system adaptations. SERT, but not NET, blockade induced CTA more quickly in MALDR than MAHDR mice, and slowed

onset of MA-induced CTA. Therefore, SERT may mediate some sensitivity to aversive effects of MA in MALDR mice. In general, this dissertation presents data indicating that voluntary MA consumption is, in part, regulated by TAAR1 function. Furthermore, behavioral and physiological studies indicate that TAAR1 increases sensitivity to aversive effects of MA, and may thereby protect against MA use.

CHAPTER 1: General Introduction

History of Methamphetamine Use

Psychostimulants have long been used for their stimulating, attention-enhancing, and euphoria-inducing effects. Dextroamphetamine, a potent psychostimulant, was synthesized by a German scientist in 1887 from its precursor, ephedrine (Anglin *et al*, 2000). The synthesis of methamphetamine (MA) by the Japanese scientist Nagai Nagayoshi followed in 1893 (Panenka *et al*, 2013). In the early to mid-1900s, amphetamines and MA were legally used both therapeutically and for recreation. As captured in era propaganda, soldiers and military pilots on both sides of World War II used amphetamine and MA to enhance wakefulness (Anglin *et al*, 2000; Iversen, 2006). Pharmaceutical production of amphetamines began circa 1930, with the production of Benzedrine™ to treat a variety of ailments ranging from asthma and narcolepsy to the common cold without prescription (Anglin *et al*, 2000; Iversen, 2006). By the 1960's, there was large scale use of MA as a prescription antidepressant, weight-loss treatment, and treatment for heroin use (Anglin *et al*, 2000).

Methamphetamine as a Drug of Abuse

It was not until 1971, a decade after the first large scale use of MA was reported, that recreational use of dextromethamphetamine, or racemic mixtures with the stereoisomer, levomethamphetamine, was outlawed in the United States (Kish, 2008). The *l*-isomer is 2-10 times less potent than the *d*-isomer, and is available over-the-counter in the widely used vasoconstrictor nasal

decongestant, *Vicks VapoInhaler*™, and other generic versions (Mendelson *et al*, 2006). However, use of either isomer is not completely without risk. Similar to the *d*-isomer, the *l*-isomer can produce cardiovascular and neurotoxic effects (discussed in detail below) when used at high enough concentrations (Mendelson *et al*, 2008), and may have potential for abuse. At 0.5 mg/kg levomethamphetamine produces similar psychoactive, intoxicating, and pleasurable effects in humans as dextromethamphetamine at the same dose (Mendelson *et al*, 2006). Throughout this document, all human dosing is converted to mg/kg or total dose, using a typical 70 kg person, to improve comparison across studies.

Popularity and availability of MA fluctuated through the 1980's and 90's in the United States. Use of MA is currently highest in the western United States and Canada, but penetrance of MA use has grown in an eastward direction across North America (Gruenewald *et al*, 2010; Maxwell and Rutkowski, 2008; Rawson *et al*, 2002). Worldwide, the United Nations Office on Drugs and Crime (UNODC) estimated that there were 25 million amphetamine-like drug users in 2006 (UNODC, 2008a), 68% (17 million) of whom were MA users (UNODC, 2008b). The UNODC estimated that 278 metric tons of MA, or 209 billion doses of 100 mg MA, were synthesized in 2005 (UNODC, 2008b). By 2009, the estimated number of amphetamine users had increased to 56.4 million (UNODC, 2011a), and between 2008 and 2013, there was a 158% increase in seizures of MA trafficked internationally (UNODC, 2015). The highest prevalence of MA use (55% of all users) is in Asia (UNODC, 2008b, 2011b). Not including alcohol and

nicotine, drugs that are legal in most places, MA is the second most abused psychoactive drug worldwide, behind only marijuana in number of users, and used by more people than cocaine and heroin combined (UNODC, 2008b, 2011b; WHO, 2014).

Using 12th grade students as a survey population, Johnson *et al*. (2015) reported that MA use reached a peak in 1999 (8.3%). Since 1999, MA use among United States 12th graders has steadily declined to only 1.9% in 2014 (Johnson, 2015). However, amphetamine use (not including MA) within this population, has remained largely steady between 1991 (15.4%) and 2014 (12.1%) (Johnson, 2015). One factor may be that prescription amphetamines, such as methylphenidate (Ritalin™) and racemic dextro- and levoamphetamine (Adderall™), are easier to acquire and are interpreted to be "safer" than illicit "street" amphetamines, such as 3,4-methylenedioxy-methamphetamine (MDMA) and MA (Green and Moore, 2009). Prescribed amphetamines, such as Ritalin™, are reportedly preferred over MA and MDMA in young Australians. Prescription amphetamine users cited fewer negative or aversive effects (such as hallucinations), and predictability of dosage and subjective effects resulting from the use of prescription amphetamines, as attractive compared to use of MA or MDMA (Green and Moore, 2009). The recent decline in MA use among high school students may also be partly a result of efforts to limit MA availability. Enhanced restriction on the availability of the primary ingredients to easily synthesize MA, such as ephedrine and pseudoephedrine, helped shift production away from "mom-and-pop" and "biker-gang" labs to "super labs" associated with

Mexican cartels (Anglin *et al*, 2000; Cunningham *et al*, 2010), and decreased MA purity (Cunningham *et al*, 2013).

Impact on Health and Communities

MA use has detrimental long-term effects on the health of users and adverse consequences throughout surrounding communities (Volkow, 2013). MA directly reduces immune system function by suppressing T-cell antigen presentation, dendritic cells, and macrophages (Talloczy *et al*, 2008). These actions likely increase a user's risk of secondary infections, such as rapid progression of human immunodeficiency virus (HIV) and bacterial infection of skin lesions resulting from formication and compulsive scratching. Additionally, cytokine, chemokine, and other inflammatory responses may perpetuate psychiatric symptoms of MA use (Loftis *et al*, 2011). Rates of HIV and hepatitis C infection are elevated in MA-using populations, likely as the result of intravenous MA use and increased risky sexual behavior (Cheng *et al*, 2010). Inhalation of MA from smoking or vaporizing may increase a user's risk of respiratory infection and severe dental damage known as "meth mouth" (Rhodus and Little, 2008), a problem likely resulting from poor oral hygiene, acidic smoke residue, and reduced salivation resulting from vasoconstriction of the salivary glands (Hamamoto and Rhodus, 2009). Additionally, MA users are at elevated risk for cardiovascular diseases such as hypertension, angina, arrhythmias, stroke, and myocardial infarction (Darke *et al*, 2008).

Cognitive deficits are well described in chronic users and may be, in part, associated with cardiovascular conditions, but are also a result of direct

neurotoxic effects of MA (Panenka *et al*, 2013). Deficits include reduced working and episodic memory, impulse control, sustained attention, and executive processes such as planning, abstract reasoning, information processing, and adaptability (Cherner *et al*, 2010; Gonzalez *et al*, 2004; Hoffman *et al*, 2006; Iudicello *et al*, 2010; Scott *et al*, 2007; Volkow *et al*, 2001; Woods *et al*, 2005). Additionally, MA-induced psychosis is often indistinguishable from acute paranoid schizophrenia (Bell, 1973). MA-induced hallucinations have been reported at 0.79 mg/kg and are predominantly auditory (experienced in 85% of cases of MA psychosis), visual, and tactile (Bell, 1973), and often are accompanied by delusions of persecution, reference, and 'mind-reading' (Chen *et al*, 2003).

Recreational use of MA has reached epidemic levels in the United States (Gonzales *et al*, 2010) and the rest of the world (WHO, 2014). According to National Institute on Drug Abuse (NIDA) statistics, abuse of alcohol, nicotine, and of illicit psychoactive substances costs Americans a staggering 550 billion dollars a year (combined medical, economic, criminal, and social impact) (NIDA, 2013). The abuse of drugs and alcohol contributes to the death of more than 100,000 Americans yearly, and tobacco abuse is linked to an estimated 440,000 deaths per year (NIDA, 2013) making substance abuse and associated complications among the leading causes of premature death in the United States.

Current Therapies for Methamphetamine Abuse

To date, there are no federally approved pharmacological therapies for the treatment of MA abuse, dependence, or withdrawal in the United States or

elsewhere (Radfar and Rawson, 2014). However, there are a number of compounds that are currently being tested or prescribed off-label. Most potential drug therapies are either neuroleptics (Shoptaw *et al*, 2009b) or antidepressants, although antidepressants may have only limited benefit to the patient for withdrawal symptoms (Shoptaw *et al*, 2009a). For example, the drugs risperidone (Risperdal™), ondansetron (Zofran™), and aripiprazole (Abilify™) have been tested as MA treatments, but failed to show promise (Coffin *et al*, 2013; Ling *et al*, 2006). This is significant because all of these drugs target monoamine systems. Modafinil (Provigil™) is a wake-promoting drug that has been approved by the United States Food and Drug Administration for the treatment of narcolepsy and sleep apnea (Ballon and Feifel, 2006). While the mechanism of therapeutic action is not well defined, administration of modafinil increased monoamine (Ferraro *et al*, 1996a, b; Stone *et al*, 2002), glutamate (GLU) (Ferraro *et al*, 1997a), orexin (Lin *et al*, 1999), and *γ*-aminobutyric acid (GABA) (Ferraro *et al*, 1997b; Ferraro *et al*, 1996b) activity in some areas of the central nervous system (CNS) of rodents and canines. Treatment with modafinil also decreased cocaine-induced euphoria (Dackis and O'Brien, 2003), and prevented MA-primed self-administration (SA) reinstatement in rats (Reichel and See, 2010). Additionally, the neuroleptics olanzapine (Zyprexa™) and haloperidol (Haldol™) appear to have mild efficacy at improving psychotic symptoms related to amphetamine use (Leelahanaj *et al*, 2005).

Bupropion (Wellbutrin™), Provigil™, naltrexone (Vivitrol™), mirtazapine (Remeron™), and baclofen (Lioresal™) are also being tested to treat MA

dependence and relapse. The effectiveness of these treatments is potentially promising, especially in combination with behavioral therapies (Radfar and Rawson, 2014). Replacement therapy has been explored in an attempt to treat MA abuse, similar to heroin dependence treatment with methadone (Dolophine™). Methylphenidate (Ritalin™) has some potential as a substitution therapy; however, the use of psychostimulants to treat MA dependence does not promote abstinence, but rather has the potential to help rehabilitate before beginning tapering or withdrawal of use.

Currently, behavioral therapies, such as 12-step or "anonymous" groups, contingency management (Roll *et al*, 2006), the "Matrix model" (Rawson *et al*, 2004), and cognitive behavioral therapy (Yen *et al*, 2004) have proven to be the most effective methods for maintenance of MA abstinence. Additionally, some laboratories have reported promising results from immunopharmacotherapies against MA use and other effects of MA in rats (Miller *et al*, 2015). For example, immunological blockade of MA action decreased MA-induced locomotor activation (Byrnes-Blake *et al*, 2001; Miller *et al*, 2013), MA-induced thermoregulatory disruptions (Miller *et al*, 2013), and MA SA (Duryee *et al*, 2009; Miller *et al*, 2015). Prevention and treatment of MA use can be improved by a better understanding of the neural mechanisms underlying risk for addiction, some of which likely contribute to sensitivity to rewarding and aversive effects of MA.

Pharmacology of Methamphetamine

While dosing patterns vary substantially among regular MA users, self-reported typical use often consists of four daily episodes of 0.7-7.1 mg/kg/episode, totaling 2.8-28.4 mg/kg/day, during binges lasting approximately 4 days (McKetin *et al*, 2006). Differences in metabolic clearance of MA in humans versus rodents complicate the comparison of self-reported dosing to the typical dosing used experimentally in rodents (Caldwell, 1976). For example, data indicate that 60% of a 0.07 mg/kg acute oral dose of amphetamine and 69% of a 0.28 mg/kg oral dose of MA was excreted in the urine of humans within 24 hours of administration, whereas 47% of a 45 mg/kg oral dose of MA was excreted by rats and 78% of an 10 mg/kg oral dose of amphetamine was excreted by mice in the same amount of time (Caldwell *et al*, 1972; Dring *et al*, 1970). The doses commonly taken by regular MA users were 2.8-28.4 mg/kg/day (McKetin *et al*, 2006), compared to doses that ranges from 0.5-8 mg/kg/day in typical rodent behavioral experiments. In rodents, doses extends to much higher (up to 30 mg/kg) for MA toxicity research (Table 1, Carmena *et al*, 2015; Phillips *et al*, 2008), with an LD50 of 57 mg/kg in mice when given acutely (Davis *et al*, 1987). Self-reported MA doses are corroborated by plasma MA levels collected in incarcerated individuals that tested positive for MA. Typical blood concentrations of MA from 105 individuals who were detained and tested positive for MA were 0.13-5.0 µM, but ranged up to 11.1 µM, which corresponds with a mean calculated MA dose of 0.74-8.6 mg/kg (Melega *et al*, 2007).

Pharmacokinetics

Absorption and Distribution

Similar to amphetamine, MA is distributed in a one-compartment model and undergoes first-order absorption (Cook *et al*, 1992). Route of administration influences the pharmacokinetics of MA, including absorption and distribution (Cruickshank and Dyer, 2009). MA bioavailability is typically highest when administered intravenously (i.v.), and decreases in availability when snorted (intranasal), inhaled (smoked or vaporized), or ingested (oral) (Cook *et al*, 1993; Cook *et al*, 1992; Harris *et al*, 2003). However, higher doses of MA may lead to higher bioavailability (Cook *et al*, 1993; Cook *et al*, 1992; Harris *et al*, 2003). One reason may be that binding of MA to receptors in the brain and periphery removes MA from the plasma compartment only to the point of binding saturation of some receptors (Cook *et al*, 1992). For example, in humans, an i.v. dose of MA produced cardiovascular effects within 2 minutes and subjective effects within 10 minutes of administration (Mendelson *et al*, 1995), and was 100% bioavailable (Cook *et al*, 1993). Peak effects occurred in less than 15 minutes (Harris *et al*, 2003), and persisted for up to 8 hours following a single moderate dose of 0.43 mg/kg (Perez-Reyes *et al*, 1991), but MA and metabolites remained detectable in plasma for several days (Harris *et al*, 2003; Mendelson *et al*, 2006).

Smoked or vaporized MA is also rapidly absorbed; however, peak plasma levels are reached more slowly than by i.v. administration and typically peak 2.5 hours after inhalation. Transfer of MA into blood may be slowed by retention of the drug in the alveoli of the upper lungs (Perez-Reyes *et al*, 1991). Existing data

indicate that bioavailability of inhaled MA ranges from 67 to 90%, and the method of inhalation (i.e. smoked or vaporized) is largely responsible for the range (Cook *et al*, 1993; Harris *et al*, 2003).

On the other hand, MA was 67% bioavailable following intranasal administration (Harris *et al*, 2003) and peak plasma concentration occurred between 2 and 3 hours (Hart *et al*, 2008). However, peak subjective effects occurred rapidly within 5–15 minutes and diminished over 4 hours (Hart *et al*, 2008). The large temporal difference in time to peak subjective effects and plasma concentration for intranasal MA may indicate acute tolerance, perhaps via redistribution of vesicular monoamines and internalization of monoamine receptors and transporters, which may limit the subjective effects of MA (Saunders *et al*, 2000; Sulzer *et al*, 2005).

Lastly, existing data indicate that oral MA ranges in bioavailability between 67 and 79% in humans, depending on the dose consumed (Cook *et al*, 1992). Peak plasma concentrations were seen 3-4 hours after oral intake of doses ranging between 0.125 and 0.25 mg/kg (Cook *et al*, 1992), 2.5 or 5 hours for 0.42 mg/kg MA (Shappell *et al*, 1996), and 5.5 or 7.5 hours for 0.14 or 0.29 mg/kg MA, respectively (Schepers *et al*, 2003). Peak plasma concentrations do not appear to correspond with dose administered to humans when MA is ingested. This could reflect differences in dose of MA administered by body weight. For example, Schepers *et al.* (2003) administered MA at 10 or 20 mg, regardless of individual body weight. Alternatively, variation could be explained by individual

physiological differences between subjects in MA absorbance, such as stomach contents or stomach pH, or individual differences in MA metabolism.

Metabolism and Excretion

The majority of MA metabolism occurs in the liver by cytochrome P4502D6. Polymorphisms in cytochrome P4502D6 may contribute to variability in MA metabolism between individuals (Caldwell, 1976; Lin *et al*, 1997). However, neither route of administration nor chronic exposure to MA appear to alter metabolic rate. Route of administration affects rate of metabolism and elimination (Cruickshank and Dyer, 2009), but, pharmacodynamic tolerance may be more influential on dose escalation during chronic or binge use in humans (Cook *et al*, 1992). In humans and rodents, metabolites of MA include amphetamine, produced by P4502D6 mediated *N*-demethylation, 4-hydroxymethamphetamine, produced by P4502D6 mediated aromatic hydroxylation, and norephedrine, produced by in noradrenergic cells by dopamine-β-hydroxylase mediated β-hydroxylation (Caldwell *et al*, 1972; Kraemer and Maurer, 2002; Lin *et al*, 1997). However, metabolites do not appear to have significant contributions to the intoxicating effects of MA. For example, metabolism of 50 mg of MA administered intranasally to humans produced a peak plasma concentration of amphetamine that was roughly 8% of the peak plasma MA concentration (amphetamine C_{max}=9.1 ng/ml *vs*. MA C_{max}=113 ng/ml), and occurred 17 hours after MA was administered, at which time acute effects are likely minimal (Cook *et al*, 1993; Harris *et al*, 2003). In mice, peak plasma levels of MA were seen about 15 minutes after an i.p. administration of 2 mg/kg MA, and decreased to baseline 4

hours after administration (Shabani *et al*, 2012b). Urine excretion of MA in humans is largely (70%) complete within 24 hours of ingestion (Cook *et al*, 1993; Cook *et al*, 1992). Urine concentration accumulates over repeated dosing; however, elimination is dependent on acidity of the urine as it can be enhanced by pharmacological acidification of the urine with oral ammonium chloride (Cook *et al*, 1993; Cook *et al*, 1992). After a typical 4-day binge, MA may be detectable in urine for up to 7 days following the termination of use (Oyler *et al*, 2002).

Pharmacodynamics

Action at Monoamine Terminals

MA is similar in structure to the monoamines norepinephrine (NE) and dopamine (DA). Much like cocaine, MA blocks membrane-bound monoamine transporters, namely the noradrenaline transporter (NET), dopamine transporter (DAT), and serotonin transporters (SERT), and blocks the reuptake of monoamines from the synapse (Fig 1.1, steps 1 and 2). However, unlike cocaine, MA is also a substrate for these transporters and reverses their endogenous function resulting in the release of monoamine transmitters into the synapse (Reviewed in Cruickshank and Dyer, 2009; Panenka *et al*, 2013; Sulzer *et al*, 2005). Thus, MA acts as an indirect agonist at monoamine receptors by causing release of DA, NE, and serotonin (5-HT) into the synapse (Fleckenstein *et al*, 2007).

MA enters the presynaptic cell by diffusion and transport. Similar to the extracellular actions of MA at monoamine transporters, once inside the cell, MA acts to devesicularize monoamines by binding to vesicular-membrane-bound

vesicular monoamine transporter-2 (VMAT-2), and reversing the function to allow the transmitter to diffuse into the cytosol (Fleckenstein *et al*, 2007). Known as the weak-base hypothesis (Panenka *et al*, 2013), devesicularization of monoamines may be, in part, due to an increase in pH of the vesicle caused by the basic pH of MA (Sulzer and Rayport, 1990). However, this hypothesis has since been largely discredited due to the extremely high concentration of MA required at the vesicle in order to increase the pH inside the vesicle (Floor and Meng, 1996; Schwartz *et al*, 2006). Once in the cytosolic compartment, the monoamine is reverse transported by monoamine transporters into the synapse and is available for postsynaptic binding. MA extends the length of activity of the monoamine transmitter by blocking NET, DAT, and SERT, and attenuating the metabolism of monoamines by monoamine oxidase (Sulzer *et al*, 2005).

Trace Amine-Associated Receptor 1 (TAAR1)

In addition to its action as a substrate for neurotransmitter and vesicular monoamine transporters (Fleckenstein *et al*, 2007), MA is a full agonist at trace amine-associated receptor 1 (TAAR1) (Bunzow *et al*, 2001; Wolinsky *et al*, 2007). Several subtypes of trace amine-associated receptors (TAARs) evolved prior to the emergence of jawed vertebrates (Hussain *et al*, 2009) and are expressed in the olfactory epithelium (Liberles and Buck, 2006). From nine initial TAARs, several more have evolved in mice, rats, and cows, while others have been lost in primates, humans, and canines (Vallender *et al*, 2010). Vallender *et al.* (2010) reported that canines express an allele of *Taar1* that codes for a non-functional TAAR1 isoform (a pseudogene). The loss of TAAR1 in dogs is

significant, because, while other TAARs are often duplicated or lost (with the exception of TAAR5), TAAR1 is stably expressed in all studied animals, except for neoteleost fish, in which it is compensated for by a similar TAAR protein (Hashiguchi and Nishida, 2007; Hussain *et al*, 2009). TAAR1 is expressed throughout the brain in human, rhesus macaque, mouse, and rat (Borowsky *et al*, 2001; Bunzow *et al*, 2001; Lindemann *et al*, 2008; Wolinsky *et al*, 2007; Xie *et al*, 2007). Relevant to this dissertation, TAAR1 is expressed in limbic structures and areas containing monoamines, including the ventral tegmental area, and dorsal raphe nucleus, and colocalized with NET, SERT, and DAT (Xie and Miller, 2008).

The 999 base pair (bp) mouse *Taar1* on chromosome 10 encodes a 332 amino acid receptor and is phylogenetically related to the 1020 bp human *TAAR1* on chromosome 6 and shares 76% homology with the 339 amino acid human receptor (Borowsky *et al*, 2001; Lindemann *et al*, 2005). There are a number of reported synonymous and non-synonymous single nucleotide polymorphisms (SNPs) in the human *TAAR1* (dbSNP, 2014), but there are no reported polymorphisms that are shared across the mouse (dbSNP, 2015) and human.

Trace amines, such as *p*-tyramine, β-phenylethylamine, octopamine, and tryptamine, interact with this G-protein coupled receptor (Borowsky *et al*, 2001; Bunzow *et al*, 2001; Lindemann *et al*, 2005; Wolinsky *et al*, 2007), and TAAR1 modulates monoamine activity, in part, through regulation of neurotransmitter and transporter availability and disposition (Revel *et al*, 2011; Xie and Miller, 2008) (Fig 1.1). Thus, TAAR1 agonists such as MA, indirectly reduce endogenous release of DA, NE, and 5-HT (Lindemann *et al*, 2008; Wolinsky *et*

al, 2007). Whereas *Taar1* knockout (KO or -/-) mice do not present an overt phenotype, they do exhibit greater spontaneous and amphetamine-induced release of NE, DA, and 5-HT in the striatum, compared to their wildtype (WT or +/+) littermates (Lindemann *et al*, 2008; Wolinsky *et al*, 2007).

TAAR1-specific agonists may have therapeutic potential to reduce MA use or addiction in humans. While prescription of TAAR1 compounds to treat human diseases is likely years away, TAAR1 agonists and antagonists have been investigated to treat a number of diseases, including schizophrenia and depression, in rodent models (Revel *et al*, 2012; Wolinsky *et al*, 2007). However, TAAR1-specific antagonists are not currently commercially available and may be limited in distribution by their inability to cross the blood-brain barrier. In rodents, pretreatment with the TAAR1 agonist, RO5263397, reduced operant SA of MA by rats (Jing *et al*, 2015). Additionally, greater locomotor stimulation to amphetamine and MA was seen in *Taar1* -/- mice compared to their +/+ counterparts (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007). Thus, TAAR1 has been implicated in MA actions at the cell and in some behavioral responses to MA, but changes in TAAR1 function have not yet been identified as a risk factor for MA use or abuse. Behavioral effects of TAAR1, such as MA-induced aversion and MA intake, require further investigation.

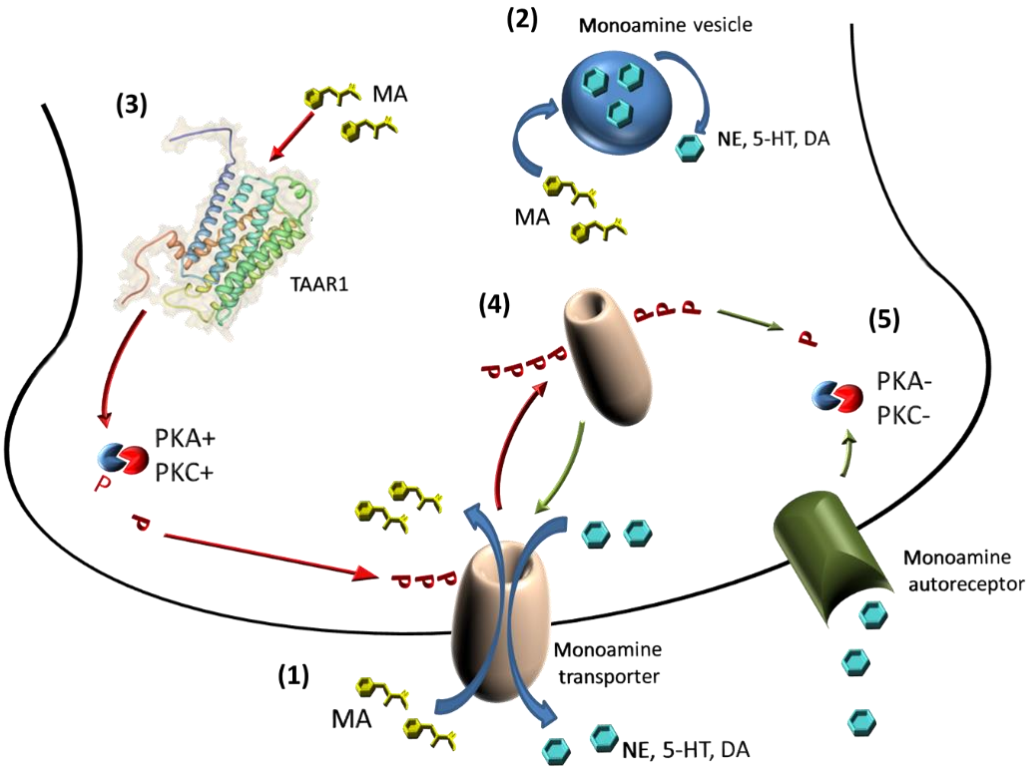

Figure 1.1 Proposed mechanism of action for trace anime-associated receptor 1 (TAAR1) regulation of monoamine activity following methamphetamine (MA) exposure. (1) MA enters the cell through diffusion or transport via monoamine transporters. (2) Once inside the cell, MA interacts with vesicular monoamine transporter 2 (VMAT2) at binding sites on monoamine vesicles, causing the release of the monoamines, norepinephrine (NE), serotonin (5-HT), and dopamine (DA), into the cytosol. Cytosolic monoamines are then reverse transported into the synapse by monoamine transporters. (3) MA and other biogenic amines bind to TAAR1, leading to the phosphorylation of monoamine transporters by the protein kinase A (PKA) and protein kinase C (PKC) pathways. (4) Phosphorylated transporters are then internalized, reducing the monoamine

uptake efficiency of the cell. (5) However, monoamine efflux is maintained by PKC-dependent dephosphorylation of remaining membrane-bound transporters. Biogenic amine binding to monoamine autoreceptors counteract receptor signaling cascades triggered by TAAR1 by dephosphorylating internalized monoamine transporters, which leads to enhanced uptake of monoamines by membrane-bound transporters.

Factors Mediating Methamphetamine Addiction

Adaptations of the Basal Ganglia Reward Pathway

The highly addictive nature of MA is, at least in part, due to positive effects experienced following use, and activation of reward pathways in the brain. Mesolimbic and mesocortical DA circuits constitute major CNS anatomy involved in drug reward and motivated behavior (Wise, 2004). MA activates “reward” circuits in the brain (Vollm *et al*, 2004). Nuclei, including the nucleus accumbens (NAcc), signal forebrain and midbrain structures via the mesolimbic and mesocortical pathways that mediate drug seeking, or “wanting;” a process that is distinct from, the rewarding effects of a drug, or drug “liking” (Robinson and Berridge, 2001). Repeated drug exposure leads to enhanced excitability of these circuits, which is partially responsible for the long-lasting and tenacious recidivism associated with drug dependence or “addiction.” Ultimately, this process of “sensitization” leads a dependent drug user to seek or “want” a drug, even as the rewarding effects of a drug diminish with repeated usage. Sensitization can be behaviorally measured by heightened sensitivity to locomotor stimulant or stereotypic effects of a drug repeatedly administered over time. It is a common response to repeatedly administered drugs of abuse, and has been demonstrated dose dependently with amphetamine, MA, cocaine, methylphenidate, fencamfamine, morphine, phencyclidine, MDMA, nicotine, and ethanol (Robinson and Berridge, 2000, 2001). The behavioral effect appears to coincide with pre- and post-synaptic changes in cell morphology. Repeated amphetamine or cocaine administration leads to increased sensitivity of

dopamine receptor 1 (D_1) and increased spine density on medium spiny neurons in the NAcc and pyramidal neurons in the prefrontal cortex (Robinson and Kolb, 1999; Wolf, 1998). Additionally, repeated amphetamine administration leads to increased amphetamine-stimulated monoamine release (Paulson and Robinson, 1995).

However, repeated exposure to higher doses of amphetamines leads to damage at DA and 5-HT terminals, which are mostly localized to the axon and synaptic terminals (Ricaurte *et al*, 1982). MA- and amphetamine-induced neurotoxicity to DA and 5-HT cells may result from the oxidation of cytosolic DA and 5-HT to 6-hydroxydopamine and 5,6-dihydroxytryptamine, which subsequently oxidize proteins and lipids in these neurons (Riddle *et al*, 2006). Additionally, elevated cerebral temperature may contribute to MA-induced neurotoxicity (Riddle *et al*, 2006).

Outside the basal ganglia, NE circuits in the hippocampus and medial basal forebrain may also be important to memory consolidation, and some rewarding effects of MA including arousal (Berridge, 2006; Berridge and Waterhouse, 2003). 5-HT signaling from the raphé nucleus has been implicated in reward signaling, hyperthermia, and sexual behavior, as well as many other functions (Hornung, 2003). However, some effects of amphetamine, including increased heart rate and elevated blood pressure, are mediated peripherally (Simpson, 1975).

Positive and Negative Subjective Effects of Methamphetamine

In addition to rewarding effects, MA has aversive effects that likely influence its use. Prevention and treatment rely on knowledge of the neural mechanisms that contribute to sensitivity to both types of motivational effects of MA and could underlie risk for addiction. An individual's propensity for repeated MA use is likely affected by the balance of their positive and negative experiences with the drug (Cruickshank and Dyer, 2009; Davis and Riley, 2010). Much attention has been given to studying the rewarding effects associated with MA addiction (e.g. Beckmann *et al*, 2010; Bryant *et al*, 2009a; Bryant *et al*, 2009b; Horton *et al*, 2011; Ikeda *et al*, 2007; Kamens *et al*, 2005b; Kelly *et al*, 2008; Meyer *et al*, 2011; Mizoguchi *et al*, 2004; Palmer *et al*, 2005; Shabani *et al*, 2012a; Shabani *et al*, 2011), but MA-induced aversion, which could limit intake, has not been given the same consideration (e.g. Pringle *et al*, 2008; Rothman *et al*, 2001; Shabani *et al*, 2012b; 2011). For example, unpleasant effects from MA and dextroamphetamine use have been reported to include nausea, headaches, fluctuating mood, depression, sleep problems, anxiety, panic attacks, and hallucinations (Green and Moore, 2009). Avoiding unpleasant effects of MA may be one reason for preference for amphetamine over MA and MDMA in some individuals (Green and Moore, 2009).

The dose of MA administered has some effect on the resulting nature of the experience (Cruickshank and Dyer, 2009); however, individual differences likely influence MA response. For example, acute improvements in attention and cognitive ability in humans have been reported at 0.43 mg/kg (Johnson *et al*,

2000; Shappell *et al*, 1996; Silber *et al*, 2006). Doses around 0.7 mg/kg have been reported to induce pleasant effects in humans, including arousal, euphoria, reduced fatigue, positive mood, relaxation, reduced appetite, and disinhibition (Harris *et al*, 2003; Mendelson *et al*, 2006; Perez-Reyes *et al*, 1991). However, doses of MA in the same range (0.43-0.79 mg/kg) may also cause dysphoric, unpleasant, or aversive effects, such as anxiety, nervousness, paranoia, hallucinations, psychomotor stimulation, increased respiration, tachycardia, and hyperthermia (Bell, 1973; Johnson *et al*, 2000; Martin *et al*, 1971; Shappell *et al*, 1996; Silber *et al*, 2006).

Several factors add complexity to understanding the subjective effects of MA. Primarily, MA affects several transmitter systems, each of which has a variety of receptor subtypes, often with complicated interactions between systems. Secondly, MA has effects in both central and peripheral systems, complicating the origin of subjects' MA experience (Cruickshank and Dyer, 2009). MA has a higher affinity for NET and produces greater release of NE than DA, and DA than 5-HT (Rothman *et al*, 2001). Rothman et al (2001) suggest that, while DA is clearly important in the reward learning aspect of MA, NE may be involved in other "subjective" (non-rewarding) effects of MA. However, baseline DA function may also influence both the positive and negative subject effects of MA administration. For example, low baseline dopamine receptor 2, ($D_{2)}$ density is associated with a pleasant response to methylphenidate, a psychostimulant similar to MA, but high baseline D_2 density predicts an aversive response (Volkow *et al*, 1999). Some data suggest that NET plays an important role in

cocaine-induced conditioned taste aversion (CTA) in mice (Jones *et al*, 2009; Jones *et al*, 2010) and rats (Serafine and Riley, 2009). Following exposure to a novel tastant, saccharin in this case, administration of a NET blocker reduced saccharin intake the next time that it was offered. Additionally, some aversive effects of cocaine may be mediated by SERT. Mice expressing null mutation for the gene encoding SERT (*Slc6a4*) have reduced sensitivity to cocaine-induced CTA, although KO of NET may have a greater effect (Jones *et al*, 2010). Given that MA causes a release of NE and 5-HT (Rothman *et al*, 2001), and that NET (Jones *et al*, 2009) and SERT (Jones *et al*, 2010) have been implicated in psychostimulant aversion, one possibility that is explored in this dissertation is that NET or SERT mediate some aversive effects of MA in the MALDR line.

Physiological Effects

Stress Axis Activation

Hypothalamic-pituitary-adrenal (HPA) axis, or stress axis, activation is one potential outcome of MA exposure. Exposure to many drugs of abuse increases HPA activity in humans and rodents. HPA activation involves increases in plasma levels of the glucocorticoids, adrenocorticotropin hormone (ACTH) and cortisol in humans or corticosterone (CORT) in rodents. Increases in plasma ACTH and CORT levels have been measured after acute treatment with MA (Grace *et al*, 2008; Szumlinski *et al*, 2001; Williams *et al*, 2000; Zuloaga *et al*, 2014). Regardless of the origin of drug action in the brain, signaling for the initiation of the HPA response converges in the paraventricular nucleus of the hypothalamus (PVN) to stimulate the release of corticotropin-releasing factor (CRF) from the

pituitary (Armario, 2006). Similar to stress-induced activation of the HPA axis, MA causes the release of CRF, which then stimulates the synthesis and secretion of ACTH from the anterior pituitary into blood (reviewed in Zuloaga *et al*, 2014). This, in turn, stimulates the synthesis and release of glucocorticoids (CORT in rodents) from the adrenal cortex of the kidneys (Armario, 2006; Dedovic *et al*, 2009).

Some data suggest that glucocorticoid release is required to sustain SA of drugs of abuse, such as amphetamine and cocaine (Deroche *et al*, 1997; Piazza *et al*, 1991), and stress exposure can initiate relapse to drug seeking (See and Waters, 2010; Shaham *et al*, 1998; Shaham *et al*, 2000; Shaham *et al*, 1997). One explanation for these results is that glucocorticoids may be important for stimulating the mesolimbic DA function that plays a role in reward (Piazza *et al*, 1996). However, elevated glucocorticoids may be experienced as unpleasant or aversive (Smotherman and Levine, 1978). In fact, administration of glucocorticoids to humans produces negative effects, such as asthenia, apathy, depression, and increased irritability (Fox and Gifford, 1953; Rome and Braceland, 1952). Furthermore, elevated plasma glucocorticoid levels have been demonstrated to enhance aversion learning in CTA and conditioned place aversion (CPA) experiments (Gorzalka *et al*, 2003; Wang *et al*, 2009). Administration of CORT may even be sufficient to induce CPA in the absence of other aversive stimuli. One study in rats found that CORT alone produced a strong statistical trend toward a CPA ($p=0.055$) that was not significantly different from the CPA seen in rats administered CORT + lithium chloride (LiCl) or LiCl

alone (Tenk *et al*, 2006). It is possible that plasma glucocorticoid levels induced by MA play a role in the aversive effects of MA. MA-induced increases in plasma CORT levels could be, in part, regulated by TAAR1, since increases in plasma CORT have been reported in rats following activation of TAAR1 by the agonists, thyronamine (T_0AM) and 3-iodothyronamine (T_1AM) (Klieverik *et al*, 2009).

Thermal Effects

In addition to HPA activation, MA exposure can alter thermoregulation in humans and rodents, resulting in hypothermia and hyperthermia. A 0.43 mg/kg dose of MA is sufficient to induce a hyperthermic response in humans (Martin *et al*, 1971). In rodents, acute and chronic MA can induce hyperthermia (Bowyer *et al*, 1994; Sabol *et al*, 2013), but hypothermia may occur at lower doses (4 mg/kg) or cooler (18–20° C) ambient temperatures (Grisel *et al*, 1997; Sabol *et al*, 2013). Propranolol, a β-adrendoreceptor antagonist, attenuated MA-induced hyperthermia, which suggests the involvement of peripheral mechanisms (Gessa *et al*, 1969). MA-induced adrenergic signaling at α_1- and β_3-adrendoreceptors in the sympathetic nervous system regulates activity and expression of uncoupling protein-3 (UCP3) and UCP1 in skeletal muscle and adipose tissue (Makisumi *et al*, 1998; Seale *et al*, 1985; Sprague *et al*, 2004). UCP activation leads to the oxidation of free fatty acids in adipose tissue, resulting in metabolically induced hyperthermia (Brodie *et al*, 1969; Gessa *et al*, 1969). However, centrally mediated mechanisms may also be responsible for regulating hyperthermia. 5-HT signaling from the raphé nucleus, as well DA activity, has been implicated in MA-induced hyperthermia (Bowyer *et al*, 1994; Hornung, 2003; Sabol *et al*,

2013), as well as preoptic hypothalamic innervations to the pituitary and release of thyroid hormone (Sprague *et al*, 2003). Thyroid hormone and NE signaling may then have a synergistic effect on UCP activation (Gong *et al*, 1997). Additionally, cutaneous vasoconstriction resulting from administration of MDMA decreases blood flow near the skin surface, likely contributing to the hyperthermic effect (Pedersen and Blessing, 2001).

Hypothermia induced by systemic or intracranial amphetamine administration has been attributed to a central DA mechanism (Kruk, 1972). For example, amphetamine-induced hypothermia in cool environments can be blocked by administration of D_2 receptor antagonists (Yehuda and Wurtman, 1972a, b). D_2 activation may affect 5-HT release and 5-HT1A-mediated hypothermia (Sabol *et al*, 2013). Similar to the involvement of DA activity in MA-induced hypothermia, TAAR1 has also been associated with hypothermic regulation, and as detailed above, has been implicated in the regulation of DA systems. TAAR1 agonists induce hypothermia (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010), including the MA-like drug, MDMA. MDMA administration resulted in hypothermia in *Taar1* +/+, but not *Taar1* −/− mice (Di Cara *et al*, 2011). Likewise, *Taar1* −/− mice did not display hypothermia to MA, but the dose of MA used in this published study did not induce hypothermia in +/+ mice (Panas *et al*, 2010), so further study is needed.

Genetic Influences on Drug Use Behavior

Many traits related to drug response and drug use are heritable and therefore associated with genotype (Crabbe, 1999). Understanding the genetic

basis of disease states can lead to better prevention, diagnosis, and treatment options for humans. In the case of drug abuse, identifying genetic correlates of predisposition may help inform at-risk individuals about their risk and thus, protect potential abusers from developing adverse use patterns. In addition, thorough understanding of the genes and mechanisms underlying heightened risk for abuse of a certain drug can guide efforts to develop effective pharmacological treatments or preventions. Although genetic tools have been used in many studies, such as single gene mutants and association studies, there is relatively little research on genetic factors that might influence risk for MA use.

Forward *vs.* Reverse Genetic Approaches

Identifying genes underlying variation in a phenotype depends on differences in gene expression or gene polymorphism that give way to variations in gene product (Palmer and Phillips, 2002). Investigation into the genetic origin of complex traits must account for gene x gene and gene x environment interactions, which can obscure the effect of any single gene (Crawley, 2007, ch. 2). Searches for gene candidates that begin at the phenotypic level (forward genetics) benefit from investigation of the whole disease state, and the use of animal models carefully chosen for their validity (Aylsworth, 1998). Alternatively, reverse genetic approaches begin at the genetic level and investigate phenotypic differences resulting from genetic modification.

Reverse genetic approaches may manipulate candidate genes by mutation, removal (i.e., KO), silencing, or replacement, and observe the

contribution of that gene to a trait of interest. For example, a line of mice can be developed that lack a particular gene of interest that has been identified for potential involvement in a phenotype. Knocking-out the gene in a mouse line excels at evaluating plausible genetic mechanisms underlying a phenotype (Palmer and Phillips, 2002), but can suffer from the global and constitutive nature of the modification and the inability to target specific tissue types or locations.

Homologous recombination is used to incorporate transgenic vectors into embryonic stem (ES) cell DNA. ES cells are then injected into blastocysts and implanted in pseudopregnant dams. Traditional KO approaches are not capable of preventing flanking DNA (genes in close proximity to the transgenic mutation) from also inserting into the ES cells during recombination and becoming integrated into the KO mice (Wolfer *et al*, 2002). Additionally, the reverse genetic approach is limited because removal of a gene or genes in a KO organism often leads to physiological or genetic compensation for the missing gene(s) (Crawley, 2007, ch. 14). This can lead to the incorrect assumption that phenotypic changes directly result from the absence of the gene product or gene effect. Mouse development and maturation in the absence of a gene may result in a number of adaptations and compensations by other genes or proteins, thus, leading to over-, and under estimation of the gene effect. For example, elimination of DAT has been investigated in DAT KO mice as a method to reduce the reinforcing effects of cocaine. However, the absence of DAT function during development led to compensation by other monoamine systems to facilitate DA signaling (Carboni *et al*, 2001; Trinh *et al*, 2003).

Developmental effects can be mitigated by using conditional or inducible transgenic methods: knockdowns, siRNAs, Morpholinos (Ferguson *et al*, 2013), Cre recombinase systems, and tetracycline-inducible systems (Graham and Self, 2010). These systems benefit from the ability to transiently turn a gene “on” or “off”, and some methods such as siRNA and Cre recombinase target specific tissues or locations. A more recent improvement in gene editing has created the ability to introduce targeted mutations with little passenger DNA. The RNA-guided CRISPR (clustered regularly interspaced short palindromic repeat)-associated Cas9 nuclease system uses guide RNA to target specific loci for removal by indel mutation, and can be used to incorporate new DNA sequences into the host genome (Shalem *et al*, 2015), giving hope to the possibility of eventual “allele swapping” in mice. This technique has the potential to eliminate concerns about flanking DNA and can be used to develop multiplexed transgenic mice, which have several introduced mutations. However, despite these developments, the forward genetic approach remains less biased and thus, advantageous, because it allows investigation of a larger polymorphic subset of genes in the normally developing context of human disease states (Belknap *et al*, 2001).

Quantitative Traits

Most behavioral and physiological phenotypes are quantitative traits – traits that may incorporate the independent and interactive effects of multiple genes and environmental factors (Palmer and Phillips, 2002). In comparison to major single gene traits, which often follow Mendelian genetic patterns, the

genetic bases of complex traits are more difficult to delineate. Subsequent to identification of phenotypic variation in a population, forward genetic investigations may narrow the location of trait-relevant genes to chromosomal regions. Statistical approaches, such as quantitative trait locus (QTL) mapping or genome-wide association study in humans, correlate genetic markers with phenotypic populations (Belknap *et al*, 1997a; Falconer and MacKay, 1996). Ultimately, the goal is to identify and validate gene candidates by the use of additional mapping, molecular genetics, pharmacological, and other tools.

A QTL is defined as a location on a chromosome containing a gene or genes with allelic variation that predicts variation in a quantitative trait (Lebowitz *et al*, 1987). Mapping can be based on the existence of known genetic markers (microsatellites, SNPs, and other polymorphisms) between two or more genetic populations, such as selectively bred lines or recombinant inbred (RI) strains. Tests for associations between genetic markers and quantitative trait scores using correlational methods can provide a log of the odds (LOD) score and indicate whether a QTL exists in the region of a marker, as well as provide a confidence interval for the QTL region (Lander and Schork, 1994; Neumann, 1992). A greater number of markers within a QTL region increases the mapping resolution, up to the point where the average intermarker and recombination intervals are equal, and reduces the number of genes within a region of investigation. However, linkage disequilibrium or chromosomal regions with fewer recombination events, will have poor QTL resolution because of fewer possibilities for allelic variation between individuals (Belknap *et al*, 2001).

Alternatively, when linkage is closer to equilibrium, examination of a greater number of genetic markers is need in order to identify closely spaced recombination sites (Palmer and Phillips, 2002). Ultimately, it has been advised that localization of a QTL into a finely mapped region of a chromosome should result in a region smaller than 1 centimorgan (cM), containing approximately 10-50 genes (Belknap *et al*, 2001; Chinwalla *et al*, 2002; Jensen-Seaman *et al*, 2004), before targeting specific genes for investigation (Belknap *et al*, 2001; Palmer and Phillips, 2002).

QTL mapping has been used to investigate the genetic underpinnings of a number of drug-related phenotypes. However, this task is not trivial. Very few quantitative trait genes (QTG) have been identified from behavioral QTL approaches, compared to the large number of QTLs that have been mapped (Mackay, 2001). Identification of *Mpdz* is one of the few examples of identification of a QTG following QTL mapping of a drug effect trait. In this case, *Mpdz* was identified as a candidate QTG for ethanol withdrawal-induced convulsions based on a QTL detected on mouse chromosome 4 (Shirley *et al*, 2004).

Alternatively, an expression QTL (eQTL) maps genomic regions associated with regulation of gene expression through analysis of thousands of gene products (Gilad *et al*, 2008). eQTLs can be used to build networks of gene regulation, linking expression patterns of relevant or similar genes to genetic "hubs" of high interest – i.e. regulation of a gene that fluctuates with the expression of many other genes (Belknap *et al*, 2013; Wheeler *et al*, 2009). Each variation of the QTL technique carries risk of incorrect identification or dismissal

of genes as candidates for further investigation (Belknap *et al*, 2001). For example, eQTL based approaches are vulnerable to missing polymorphic effects that do not alter gene expression (discussed in detail in Chapter 4).

QTL Mapping Populations

Crosses derived from inbred strains. Inbred strains of mice are useful for the investigation of genetic influences on behavior because of their genetic uniformity within a strain, and consistent genetic differences between strains. After 20 generations of sibling pair mating, same-sex individuals from inbred strains are approximately 98.7% genetically identical, and homozygous at virtually all loci (Crabbe, 1999; Falconer and MacKay, 1996; Silver, 1995). At this point, phenotypic variation within an inbred strain is mostly due to environmental influences. Although these strains are largely genetically and phenotypically stable over many generations and locations (Crabbe, 1999; Falconer and MacKay, 1996), random mutations arising from subsequent breeding limiting absolute homozygosity (Crabbe, 1999).

For example, C57BL/6J (B6) inbred mice are known to stably drink high levels of ethanol, whereas DBA/2J (D2) inbred mice will not (Crabbe, 1999; Yoneyama *et al*, 2008). These mice also reportedly differ in their consumption of MA, although only one study has been published examining this in these two strains. In this study, D2 mice consumed greater amounts of MA than B6 mice (Eastwood and Phillips, 2012). These inbred strains have also been tested for other MA traits: for example, the D2 mice are very sensitive to MA-induced locomotor sensitization (Phillips *et al*, 1994). Variation in phenotypic scores

between inbred strains may indicate genetic influence on the phenotype and allelic variation between the strains. However, two inbred strains that differ in phenotypic scores cannot be simply compared to conclude genetic influences on trait scores (Crabbe *et al*, 1990). Often strains will be genetically distinct at many loci, only some of which are important to the trait in question. Thus, genetic correlations collected from small numbers of inbred strains cannot identify correlated genes. However, panels of inbred strains can improve the reliability of genetic correlations through increased power to detect genetic variation common to trait relevant loci (Crabbe *et al*, 1990; Palmer and Phillips, 2002).

Calculations of heritability (h^2), or the additive genetic variance that contributes to individual differences in phenotypic score, can be used to estimate the genetic contribution to phenotypes that arise across inbred strains. Polymorphisms and microsatellites that differ among inbred strains, or crosses derived from inbred strains, can be used to identify regions of chromosomal and genetic influence on a trait. RI strains are a special panel of inbred mice that provide a powerful tool for examination of genetic influences on complex traits, which is exemplified by their use in addiction research to investigate the genetic basis of a number of drug responses (Grisel *et al*, 1997; Phillips *et al*, 2014; Phillips *et al*, 2008). RI strains or congenic strains of mice that possess regions of particular chromosomes, isolate regions of chromosomes that correlate with phenotypic variation and can identify hotspots for further investigation (Palmer and Phillips, 2002).

One example of a panel of RI strains in addiction research has been the BXD panel of RI strains. This panel was derived by inbreeding multiple strains of mice beginning with pairs of mice from the F2 cross of the B6 and D2 inbred strains (B6D2F2). These strains have been used in many mapping studies for complex behavioral and physiological traits, including MA-induced traits like hypothermia and hyperthermia (Grisel *et al*, 1997). Unfortunately, the BXD RI panel is limited to the genetic differences, and also phenotypic differences, that exist between the B6 and D2 inbred strains. A total of 102 BXD strains have been fully inbred and about 81 of these are currently available from The Jackson Laboratory, with newly derived BXD strains currently being developed (Gene Network, 2015). However, mapping approaches based on inbred or RI mouse backgrounds are limited by the number of inbred stains available, and thus, statistical power to detect QTLs (Churchill *et al*, 2004), which results from low genetic diversity arising from genomic overlap among most inbred mouse strains (Roberts *et al*, 2007).

Genetic diversity captured in outcrossed lines is benefitted by increased allelic variation from multiple originating populations (Flint *et al*, 2005), potentially improving genetic correlations with phenotype. For example, the Diversity Outcross (DO) and Collaborative Cross (CC) heterogeneous stock mouse populations provide high genetic diversity, which allows for finer mapping precision and resolution due to a high frequency of recombination events (Chesler, 2014; Gonzales and Palmer, 2014). The CC was derived from an 8-way cross of inbred mouse strains (Churchill *et al*, 2004; Threadgill *et al*, 2011),

and has the goal of retaining polymorphisms every 100 to 200 bp. However, finer mapping resolution comes at the cost of limiting the power to detect small effect size QTLs due to the increased number of independent tests needed as a result of the number of markers, requiring a more stringent alpha to meet statistical significance. These advanced genetic models provide some advantages similar to standard inbred mouse strains, including their genetic and phenotypic reproducibility, and use of data repositories, such as the Mouse Phenome Database (Bogue and Grubb, 2004; Grubb *et al*, 2004), to share phenotypic and genotypic data (Churchill *et al*, 2004).

Selectively Bred Lines

To avoid some limitations of inbred strains, mice can be selectively bred for a particular phenotype. Artificial selection is well established as a method to develop agriculturally beneficial varieties of crops and livestock (Flori *et al*, 2009). Breeding two individuals together that share a phenotype will produce offspring that are more likely to have a similar degree of that phenotype than breeding of random individuals from the population (Crabbe, 1999). Thus, multiple generations of selection for a particular phenotype has the effect of increasing the frequency of phenotype-associated alleles in the selected population (Falconer and MacKay, 1996). However, selective breeding will be successful only for traits with at least a partially additive genetic basis (Crabbe, 1999).

Selective breeding requires a genetically heterogeneous starting population. Knowledge of differential MA intake between the B6 and D2 strains and differences among BXD RI strains (Belknap, unpublished) led to the decision

to create selected lines based on high and low levels of voluntary MA drinking to use as a mapping population and examine additional phenotypes, as described in detail below. Additionally, several other mouse lines selected for drug-related traits have been derived from B6 and D2 inbred strain crosses (Atkins *et al*, 2001; Kamens *et al*, 2005a; Scibelli *et al*, 2011; Shabani *et al*, 2011; Wheeler *et al*, 2009). Similarly, a heterogeneous founding population can be developed from a larger number of mouse strains, for example, as described above for the CC. Several drug-related selective breeding projects have been initiated from multistrain crosses (Churchill *et al*, 2004; Threadgill *et al*, 2011).

Often, lines are bidirectionally selectively bred for high scores in one line and low scores in the other (e.g., Atkins *et al*, 2001; Scibelli *et al*, 2011; Shabani *et al*, 2011; Wheeler *et al*, 2009), but unidirectional selection can also be used (e.g., Crabbe *et al*, 2009; Crabbe *et al*, 2010). There exists a challenge to limit inbreeding of non-trait relevant genes while breeding for selected traits, with practical limitations in breeder availability and resources needed for large numbers of breeding pairs (Crabbe, 1999; Falconer and MacKay, 1996). Potential segregation of genes not relevant to the selection trait, due to inbreeding or random drift, must be considered when interpreting the results of correlated trait studies. Correlated trait studies could include examinations of other behavioral phenotypes or of genetic, neurochemical, or physiological differences. Additionally, rare allele types are unavoidably lost during selection and often during the first generation. These “founder effects” and “genetic drift” can limit genetic variability within the population (Crabbe *et al*, 1990). For these

reasons, selection for a phenotype should be replicated in order to verify the reproducibility of previous selection and correlated trait findings (Crabbe *et al*, 1990).

Several selective breeding strategies exist. The two most often used methods have been individual selection and family selection. In mass selection (aka individual selection), the most extreme scoring males and females from each generation, regardless of family, are mated together to form the high or low scoring breeding populations for that generation. This method results in rapid phenotypic selection, but extreme scoring individuals tend to be more closely related, and therefore mating results in higher levels of inbreeding (Crabbe, 1999). It is often possible to reduce inbreeding by explicitly avoiding the breeding of close relatives, especially siblings.

Family selection can be divided into between-family and within-family selection approaches (Falconer and MacKay, 1996). The main difference between these methods is the number of animals selected from each family, and the selected breeders that are paired. Between-family selection tests all individuals, and then entire families are kept as breeders based on high or low family mean phenotypic scores. While between-family breeding is also prone to high levels of inbreeding, like mass selection, between-family selection is able to quickly select for traits (or for traits with low heritability) by mating extreme scoring siblings (Blair *et al*, 1989). Within-family selection also tests the phenotype of all individuals, but only selects the most extreme scoring male and female member of a family to be used as breeders for subsequent generations.

The benefit of within-family selection is lower susceptibility to inbreeding because individuals are bred to individuals outside of their family (Crabbe, 1999).

Common to all types of selection, and discussed previously for inbred strains, is the problem of gene fixation and genetic drift. Short-term selection uses mass selection to drive phenotypic segregation, but this results in greater inbreeding, so selection is limited to a smaller number of generations (1-5 selection generations), thereby limiting the accumulated inbreeding (Belknap *et al*, 1997b).

Once selection has been completed, several metrics of selection response and heritability can be calculated. The h^2 can be estimated from the realized response to selection pressure regressed on the selection differential. The realized response can be calculated for the entire selection, or for earlier selection generations, when response is likely to be greater, especially with mass selection. Realized response to selection pressure is generated from the difference between the mean trait scores of the selection generation and the founding population (Belknap *et al*, 1997a; Falconer and MacKay, 1996). Similarly, the selection differential describes the difference in mean phenotypic scores between the breeders selected to produce the next generation and the population from which they were selected.

When the response to selection has ceased, additive genetic variance has typically been exhausted, due to fixation of trait-relevant genes (Crabbe, 1999). However, heterozygosity may remain in the case of dominance, when a trait is similar for animals that are heterozygous or homozygous for a particular allele

form (e.g., Phillips *et al*, 2002). As described above, following the completion of phenotype selection, the location of trait-relevant genes may be narrowed to chromosomal regions by QTL mapping. Potentially, gene candidates will be identified and validated by the use of additional mapping, molecular genetics, pharmacology, and other tools.

Behavioral genetic investigations of drug abuse have utilized selective breeding for many drug-related phenotypes. However, by far, the majority have been for ethanol-related traits, such as mouse lines selectively bred for two-bottle choice ethanol intake (Belknap *et al*, 1997b; Crabbe *et al*, 1990). These selections include two replicates of high-alcohol preferring (HAP) and low-alcohol preferring (LAP) mice (Grahame *et al*, 1999), two replicates of high drinking in the dark (HDID) mice (Barkley-Levenson and Crabbe, 2014, 2015; Crabbe *et al*, 2009; Crabbe *et al*, 2010), the Indiana alcohol preferring (P) and non-preferring (nP) rats (Limeng *et al*, 1977), the alcohol-preferring AA (Alko, Alcohol) and alcohol avoiding ANA (Alko, Non-Alcohol) strains (Eriksson, 1968), the Italian Sardinian ethanol preferring (sP) and non-preferring (sNP) lines (Colombo *et al*, 2006), and the UChA and UChB strains (Mardones and Segovia-Riquelme, 1983), among others. Selective breeding has been far less often used for MA-related traits, but bidirectional selective breeding has been used to create high and low lines for MA activation (HMACT and LMACT) (Kamens *et al*, 2005a), higher and lower levels of MA-induced locomotor sensitization (MAHSENS and MALSENS) (Scibelli *et al*, 2011), and sensitivity to MA-induced stereotypy (Atkins *et al*, 2001). Specific to the work described here, selective breeding has been

used to create lines that differ in two-bottle choice consumption of MA (Shabani *et al*, 2011; Wheeler *et al*, 2009).

The High and Low MA Drinking (MADR) Selected Mouse Lines. Two replicate sets of mouse lines that were bidirectionally selectively bred for amount of MA consumption have been fully described (Shabani et al, 2011; Wheeler et al, 2009). The high and low MA drinking (MADR) lines were selectively bred on the basis of voluntary MA intake in a two-bottle choice procedure, in part, because voluntary drinking in this model provides a method to measure the amount of drug consumed and preference for the drug-containing solution (Sanchis-Segura and Spanagel, 2006; Scibelli et al, 2011; Shabani et al, 2011; Wheeler et al, 2009), and can accommodate testing many mice simultaneously in a relatively short procedure. Other models of drug intake, such as SA, require longer periods of training and are restricted in the number of mice that can be tested by equipment availability, and other factors. These models are described in greater detail below.

Briefly, MADR lines of mice used here were derived from the F2 cross of the B6 and D2 inbred strains, and selected using a short-term mass selection procedure. Mice were isolate housed and habituated for 48-hour to drinking from two 25 ml drinking cylinders fitted with sipper tubes, containing water. During days 3-10, mice were offered 25 ml drinking tubes containing tap water vs. MA (20 mg/l for 4 days, then 40 mg/l for 4 days). Mice had 18-h access to a MA-containing tube, which was removed during hours 3 through 9 of the light cycle. Water tubes remained available 24 h/day. The positions of the tubes were

alternated every 2 days to control for side preferences. Consumption averages from the second and fourth days of each MA concentration were used to calculate consumption (MA and water) and preference ratio (18h MA consumption : 18h total fluid consumption). Body weight was measured every 2 days and used to calculate amount of MA consumed in mg/kg. (Shabani *et al*, 2011; Wheeler *et al*, 2009). Initially, a founding population of 120 F2 mice was tested for amount of MA consumed. The 13 highest and lowest male and female breeders were selected based on their consumption (mg/kg) of the 40 mg/l solution to establish the MA High Drinking (MAHDR) and MA Low Drinking (MALDR) lines. Selection in subsequent generations selected the 13 highest and lowest male and female offspring to be breeders within the respective lines.

Calculation of realized h^2 for the two completed selections indicated that ~35% of the variability in MA intake can be attributed to genetic differences (Shabani *et al*, 2011; Wheeler *et al*, 2009). The majority of line differences in MA intake occurred by the second selection generation (Shabani *et al*, 2011; Wheeler *et al*, 2009), indicating reduced variance of MA-drinking relevant genes as selection progressed, and likely rapid fixation of some trait relevant alleles. These selected lines provide a strong animal model for investigation of genetic influences and mechanisms associated with MA intake, and for identification of genetically correlated phenotypes.

Differences in neural responses to MA likely influence MA intake and may regulate differences in MA sensitivity between the MADR lines. However, basic physiological responses to MA have not yet been characterized in these mouse

lines and could influence risk for MA consumption. For example, MA is known to produce hyperthermia in humans, which under certain conditions, is associated with toxicity (Matsumoto *et al*, 2014). In addition, HPA activation may be an outcome of MA exposure that is related to MA-induced aversion. Increases in plasma ACTH and CORT levels have been found in rodents after acute treatment with MA (Grace *et al*, 2008; Szumlinski *et al*, 2001; Williams *et al*, 2000).

Methods to Study Drug Effects in Animals

The use of animal models in addiction research allows for certain observational methods, types of experimentation, and control over drug history that human research does not allow. Mouse models of psychostimulant intake and seeking provide powerful tools to study principles of human psychostimulant dependence and addiction. Furthermore, manipulation and investigation of genes associated with behavior and physiology are possible in rodents that cannot be matched in humans. It is, therefore, convenient that mice and rats will voluntarily consume, or work to consume, many drugs of abuse. Furthermore, rodents share a large degree of similarity with human neural and peripheral physiology, and psychology. Unfortunately, rodents are ineloquent at best and cannot model every aspect of human addiction or drug abuse. Thus, animal models of drug consumption and drug seeking require exacting consideration of the experimental design and analysis to study motivation and interpret behavior. Furthermore, there are models that take advantage of Pavlovian conditioning such as conditioned place preference (CPP) and CTA procedures.

Two-Bottle Choice Drinking

Gustatory behaviors are natural behaviors for a rodent and will be performed without experimenter intervention. The two-bottle choice drinking procedure incorporates measurement of response for an unconditioned reinforcer, which is used as a metric of preference; in this case measuring the consumption of a drug solution in comparison to consumption of water. Voluntary drinking in this model provides a method to measure the amount of drug consumed and preference for the drug-containing solution (Sanchis-Segura and Spanagel, 2006; Scibelli *et al*, 2011; Shabani *et al*, 2011; Wheeler *et al*, 2009). Consumption of the MA solution, or preference for MA over water, is interpreted as "liking" the solution. MA consumption by the MADR lines has been published for the first and second replicates of selection that were completed before the time that this dissertation work was being performed (Shabani *et al*, 2011; Wheeler *et al*, 2009). Each of these selections produced remarkably similar results for MA consumption, with MAHDR mice consuming between 6 and 7 mg MA/kg/18 h (40 mg/l MA), and MALDR mice consuming about 0.5 mg MA/kg/18 h on average by the last generation of selection.

The advantage of this procedure is the ability to assess individual differences in voluntary oral drug administration, and the lack of training necessary for the animal to perform the procedure. Additionally, the relatively short amount of time needed to conduct the procedure and the ability to simultaneously test many animals make two-bottle choice drinking a high-throughput procedure. For these reasons, two-bottle choice drinking has been

used to develop many selectively bred lines of mice and rats for ethanol consumption, as described above. Strengths of this procedure also include that a rodent is given the choice of ad lib consumption of the drug solution, but has the ability to satiate thirst by consuming water and is therefore not obligated to consume the experimental solution. Additionally, choice drinking is considered to be a good model of human drug intake. While oral ethanol intake is often described in humans and thus translates well to rodent models, MA (Cook *et al*, 1993; Cook *et al*, 1992; Harris *et al*, 2003) and cocaine (Oliveto *et al*, 1995; Van Dyke *et al*, 1978) also produce similar physiological and subjective effects between humans and rodents when administered orally or otherwise.

However, this procedure is not without challenges. Consumption of a drug solution can be affected by sensitivity to the drug's effects. Differences in sensitization may be genetically associated with differences in MA consumption (Shabani *et al*, 2011). Extreme sensitivity to MA may play a protective role against psychostimulant drug intake. For example, the HMACT and LMACT lines were tested for MA and cocaine locomotor response and consumption. LMACT mice consume more MA and cocaine solutions than HMACT mice, but had lower locomotor stimulation to either drug (Scibelli A. C. *et al.*, 2011). Acute or sensitized stimulation can measure drug sensitivity and has been investigated in the MADR lines for MA administration (Eastwood and Phillips, 2012; Olsen *et al*, 2013; Shabani *et al*, 2011), as well as cocaine (Gubner *et al*, 2013). The lines have been found to be largely similar in their locomotor response to several low

to moderate doses of these drugs, but these results are discussed in detail in Chapter 4.

Drug-induced locomotor effects, such as sedation, can also alter consumption not due to drug preference. Monitoring of total volume consumed in relation to drug consumption can help control for this possibility. Additionally, fluid consumption may be influenced by perception of, or inability to perceive, the taste of the solution. For example, MA is reported to have a bitter taste (NIDA, 2015). One possibility for diverging MA intake in the MADR lines is sensitivity or insensitivity to bitter taste perception, rather than sensitivity to the pharmacological effects of MA. Therefore, the MADR lines have been tested for consumption of psychoactively inert tastants including, sweet, salty, and bitter flavors (Shabani *et al*, 2011; Wheeler *et al*, 2009). Overall, the lines do not differ in consumption of tastants, which is discussed in detail in Chapter 4. Lastly, solution consumed by the animal can be interpreted as preference or non-preference for the drug, but can be complicated by titration of drug consumption in order to avoid overdose. For example, an animal can drink high levels of an ethanol solution and still consume enough water for survival because ethanol concentrations are usually kept low. However, it may not be possible for an animal to consume only MA solution at a level required for hydration without overdosing and thus, must also consume water. The result may be that MA : water consumption ratios do not indicate “preference”, but this does not indicate that the animal does not like or want the drug. Thus, it is important to interpret MA preference relative to other experimental groups.

Self-Administration

Similar to two-bottle choice drinking, several important characteristics of drug reward can be investigated by measuring the degree to which a rodent will work for a drug or drug-paired cue. Drug SA operantly reinforces responses by a rodent on a manipulandum or instrument with drug administration, contingent paired cue (lights, tone, etc.), or combination thereof, which can be used to determine an animal's motivation to obtain the reward (Tabakoff and Hoffman, 2000). Operant SA procedures are considered to be a valid model of drug taking in humans. This method is often used to investigate the "reinforcer efficiency" of a drug by measuring its ability to maintain responding (Griffiths *et al*, 1979). Similar to two-bottle choice consumption, the MADR lines diverged in intracranial and oral operant SA of MA (Shabani *et al*, 2012a). Operant SA of oral MA in the MAHDR, but not MALDR, line indicates elevated motivation for MA seeking in the MAHDR line.

Different operant procedures can be used to delineate different characteristics of the reinforcer and internal state of the animal. Fixed ratio and fixed interval schedules of reinforcement deliver a reinforcer following a fixed number of responses, or a fixed period of time between responses. Fixed schedules measure rewarding pharmacological effects of a drug, and are widely used in favor of more complicated schedules of reinforcement (Richardson and Roberts, 1996; Sanchis-Segura and Spanagel, 2006). On the other hand, progressive ratios of reinforcement avoid anticipatory effects of responding and can be used to investigate effectiveness of a reinforcer by progressively

increasing the threshold required for the animal to receive a reinforcer. Thus, break-points in responding, when the responses required no longer elicit responding by an animal, indicate the relative value of the drug, or drug seeking, that cannot be measured by fixed-ratio reinforcement (Panlilio and Goldberg, 2007). At this time, MADR mice have not been studied using variable schedules of reinforcement, but research is ongoing.

Operant SA procedures generally require complicated or lengthy training procedures, which can be unfeasible when high-throughput is needed. Furthermore, reductions in responding can be complicated by unintended side effects of pharmacological treatments (such as sedation or other impediment to responding) that do not reflect a reduction in motivation by the rodent. Lastly, while the particular manipulandum or response is typically chosen to be a normal behavior for the rodent (such as nose poking), the behavior is none-the-less trained and not naturally occurring outside of the operant chamber. These complications, and particularly the need to test a large number of mice, excluded operant SA as a choice for selective breeding.

Conditioned Place Preference and Place Aversion

As discussed above, operant SA procedures investigate reinforcing effects of a drug and two-bottle choice procedures investigate preference for drug intake versus water. Conditioned place preference (CPP) procedures measure the preference an animal has for an environment paired with a drug state, a response that is interpreted as a metric of the rewarding subjective effects of a drug (Cunningham *et al*, 2006a). CPP apparati are typically chambers divided

into two or three compartments. In the 3 compartment model, one compartment remains neutral, whereas the other 2 are used to condition an association with saline and drug (Tzschentke, 2007). The two conditioning compartments may be designed to be equally preferred or initial preference may be measured and considered in the effect of drug conditioning (Cunningham *et al*, 2006b; Tzschentke, 2007). In an unbiased procedure, the compartment used for drug conditioning may not consider initial preference or the chambers may be matched for initial preference. In a biased procedure, the least preferred compartment for a particular animal is used as the drug-paired compartment, which increases the probability that a preference will be conditioned (Tzschentke, 2007). Based on classical Pavlovian conditioned approach, the CPP procedure pairs an injection of a drug, the unconditioned stimulus (US), with one environment or context, the conditioned positive stimulus (CS+). Environmental cues can consist of tactile flooring types (grid vs. hole), but can also be visual cues such as floor or wall pattern (Cunningham *et al*, 2006b). Vehicle is administered prior to placement in a second environment or context, the conditioned negative stimulus (CS-). Association of the drug effect is paired with environmental cues (Cunningham *et al*, 2006a; Cunningham *et al*, 2006b). Over repeated pairings, the animal is conditioned to associate a particular environment with a drug state. Motivational valence of the US (conditioned response) can be assessed by preference for the drug (CS+) or vehicle paired (CS-) environments (unconditioned response). Time spent in the CS+ environment, or entries into the CS+ environment, are interpreted as preference for the rewarding effects of the

drug (Cunningham *et al*, 2006a). Additionally, testing can occur in either a drug-present or drug-absent state (Tzschentke, 2007). Testing in drug-absent conditions specifically measures drug-cue discrimination; however, testing in a drug-present condition invokes interoceptive processes similar to state-dependent learning, and may increase the expression of aversive hedonic values conditioned to the paired floor (Cunningham and Noble, 1992).

Although CPP was not chosen as the test used for selective breeding, the MAHDR line has consistently been found to be more sensitive to the rewarding effects of MA in CPP tests. Wheeler *et al.* (2009) and Shabani *et al.* (2011) demonstrated CPP in replicates 1 and 2 of the MAHDR line, but not in the MALDR line for 0.5 mg/kg MA, using the drug-free testing procedure. In replicate 2, doses of 0.5, 2, and 4 mg/kg MA were tested and the line difference was not dose-dependent (Shabani *et al*, 2011). There was no significant preference for either the paired or unpaired floor in MALDR mice, indicating that they conditioned neither a preference nor an aversion to MA in this procedure. In the drug-present test, CPA in the MALDR line was not dose-dependent for doses of 0.5, 2, and 4 mg/kg MA, and CPP in the high line was seen at the lowest (0.5 mg/kg) dose tested, when examined separately from other doses.

A slightly different approach can be used to measure CPA, or avoidance of a place paired with a drug state. More robust CPA has been demonstrated when drug is administered *following* exposure to a particular (paired) environment (Cunningham *et al*, 2003). Paradoxically, some drugs (for example, ethanol) will condition CPP at some doses or via some routes of administration,

in some species, but induce CPA in others (Cunningham *et al*, 1993). For example, when MA was administered following context presentation, MALDR mice exhibited robust CPA at 2 and 4 mg/kg MA, but not 0.5 mg/kg, and MAHDR mice exhibited CPA after conditioning with 4 mg/kg MA (Shabani *et al*, 2012b). Pairing MA following exposure to the context produces association of aversion with the paired context, similar to the procedure used to induce CTA (discussed below). One theory of temporally-dependent paradoxical drug effects suggests that contextual associations form, based on the drug effect that is first experienced (Shabani *et al*, 2012b). Exposing mice to MA immediately following the context forms an association with the initial aversive effects of MA during the ascending arm of intoxication. However, MA exposure prior to the context prolongs the duration during which context/MA associations can be made, and thus, there is greater exposure to the pleasant effects of intoxication (Cunningham *et al*, 2003; Shabani *et al*, 2012b).

Strengths of the CPP and CPA procedures include the simplicity of animal response and the lack of operant training required. However, the procedure is not without some weaknesses. For example, initial floor biases can complicate interpretation of data. Solutions for initial preference are usually either to randomize floor type pairings (CS+ or CS-) and balance rodent preferences across pairing groups. Alternatively, “biased procedures” assign CS+ floor type based on lesser initial floor preference in order to work against bias when conditioning place preference. Additionally, animal attention or perception is a concern during conditioning. For example, if using visual rather than tactile cues,

it is possible that an animal may be attending to the preferred environment while being physically in the other (Cunningham *et al*, 2006b).

Conditioned Taste Aversion

CTA pairs the perception of an aversive effect with a taste (Cappell H. & LeBlanc A. E., 1971). It is advantageous for an animal to learn to identify substances that can induce illness when consumed, and may therefore threaten survival or reproductive fitness (Sanchis-Segura and Spanagel, 2006). CTA is a highly conserved process that relies on well-described neural circuitry, including the nucleus of the solitary tract (NTS), pontine parabrachial nucleus, and central amygdala (CeA) (Bahar *et al*, 2003; Bernstein and Koh, 2007; Chambers, 1990; Rinaman and Dzmura, 2007). Furthermore, conditioning of CTA can be blocked by elimination of NE activity in the NTS and CeA (Bahar *et al*, 2003; Rinaman and Dzmura, 2007). Rinaman et al. (2007) suggest that NE is responsible for hypophagia and stress associated with CTA learning. In experimental settings, this learning process can be leveraged to determine the aversive nature of a drug that is not dependent on context or paired cues. For example, a rodent is allowed to drink a preferred solution, such as a novel sweet saccharin or sucrose solution, or a salty sodium chloride solution, immediately prior to an injection of a drug or vehicle (Sanchis-Segura and Spanagel, 2006). Over several pairings, aversive drugs will reduce consumption of the paired solution, while administration of the vehicle or a non-aversive drug will not (Bures *et al*, 1998).

Differences in MA intake in the 2-bottle choice procedure could involve differences in sensitivity to MA-induced CTA. Thus, this trait has been examined

in the MADR lines. The MADR lines reliably differ in sensitivity to MA induced CTA. The MAHDR line does not respond to MA-induced CTA at any dose tested, up to 4 mg/kg. MALDR mice, on the other hand, are highly sensitive to MA-induced CTA at every dose tested, as low as 1 mg/kg (Shabani *et al*, 2012b; Wheeler *et al*, 2009). This again exemplifies the consistent sensitivity to the aversive effects of MA seen in the MALDR line.

One disadvantage to the CTA procedure is that animals must be water deprived prior to, and during, the drug-pairing procedure to ensure high initial consumption and motivation to consume the paired solution (Riley, 2011). However, this weakness can also be interpreted as a strength of the procedure, because decreased consumption of the paired solution, despite water deprivation, is a demonstration of the animal's motivation to avoid the paired solution and of the strength of the aversion. Water deprivation and resulting decreased food intake can have detrimental health effects, which must be monitored by measuring body weight and observing the general activity of the rodent.

QTL Mapping and Gene Expression Analysis in the MADR lines

A QTL on mouse chromosome 10 accounts for greater than 50% of the genetic variance in MA intake in MADR mice (Belknap *et al*, 2013). Additionally, QTLs that account for smaller amounts of the genetic variance have been mapped to locations on other chromosomes. The chromosome 10 QTL has a confidence interval of 10-40 Mb on the proximal end of chromosome 10 (Belknap *et al*, 2013) a region that could contain as many as 1,500 genes, assuming 1 cM

contains about 10-50 genes (Belknap *et al*, 2001; Chinwalla *et al*, 2002; Jensen-Seaman *et al*, 2004). The lack of polymorphic markers near the centromere, make the exact interval somewhat more difficult to define. Finer mapping of the chromosome 10 QTL had not yet been completed, but *a priori* hypotheses targeted several candidate genes that reside within the QTL region. These include the μ-opioid receptor gene (*Oprm1*), metabotropic glutamate receptor gene (*mGlur1*), and *Taar1*, which reside at 6.76, 10.7 and 23.9 Mb, respectively. At the time this dissertation project was designed, work had begun to study the potential role of *Oprm1* and μ-opioid receptors in MA intake. It is possible that *Oprm1* regulates a portion of MA drinking. Specifically, a network analysis of risk for MA intake identified *Oprm1* as a significant hub when it was added to the risk network. *Oprm1* was regulated by multiple genes in that risk network (Belknap *et al*, 2013); thus, it may be a downstream target, rather than a direct predictor of risk for MA use. The MADR lines significantly differ in both opioid sensitivity and intake. Among other findings discussed in detail below, MALDR mice consume greater amounts of morphine (Eastwood and Phillips, 2014), and MALDR mice exhibited greater acute stimulation than MAHDR mice to morphine and fentanyl, two μ-opioid receptor agonists (Eastwood and Phillips, 2012).

MAHDR mice also have greater expression in the NAcc of the genes encoding NET (*Slc6a2*) and SERT (*Slc6a4*), but not dopamine (DAT) transporters than MALDR mice (Wheeler *et al*, 2009). These genes do not fall within the chromosome 10 QTL (Belknap *et al*, 2013; Wheeler *et al*, 2009), but are targets for MA action and have been implicated in the aversive effects of

cocaine (Jones *et al*, 2009), and physiological processes such as thermal regulation (Gong *et al*, 1997; Sprague *et al*, 2003). Subsequent to the beginning of my dissertation work, but important to understanding monoamine involvement in MADR differences, Lominac *et al.* (2014) reported higher expression of DAT and SERT, but lower expression of D_2 receptors in the NAcc core of MAHDR mice, compared to MALDR mice. Higher expression of DAT, but lower expression of 5-HT1B, was also found in the NAcc shell of MAHDR mice compared to MALDR mice. Additionally, MAHDR had a trend of higher 5-HT1B expression than MALDR mice in the mPFC.

Another reason for interest in the difference in transporter expression in the MADR mice, is because TAAR1 modulates monoamine transporters in mice and primates (Miller, 2011, 2012; Revel *et al*, 2011; Xie and Miller, 2008, 2009). Furthermore, D2 mice, one of the progenitor strains to the MADR selected lines, possess a unique non-synonymous single nucleotide polymorphism (SNP, C229A) in *Taar1* (Sanger Mouse Genome Project SNP Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011) that is not present in B6 mice, the other progenitor strain. In combination, this evidence made *Taar1* a strong genetic candidate for further investigation.

Taar1 Knockout Mice

KO and other transgenic mice can be beneficial to exploration of gene candidates for a trait. KO mice were used in my dissertation project to investigate *Taar1* involvement in MA intake in the MADR lines.

As described above, MA is a agonist for TAAR1 (Bunzow *et al*, 2001;

Wolinsky *et al*, 2007), and TAAR1 agonism is associated with some effects of MA, including operant SA of MA in rats (Jing *et al*, 2015), and locomotor stimulation to amphetamine and MA (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007). Additionally, MAHDR and MALDR mice have differences in monoamine transmitter activity and transporter expression (Lominac *et al*, 2014; Wheeler *et al*, 2009), both of which may be influenced by TAAR1 activity (Lindemann *et al*, 2008; Revel *et al*, 2011; Wolinsky *et al*, 2007). Given this information, along with the non-synonymous SNP in *Taar1* of D2 compared to B6 mice (Sanger Mouse Genome Project SNP Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011), and the location of *Taar1* within the confidence interval of the chromosome 10 QTL, investigation of *Taar1* as a viable candidate for a QTG regulating MA intake was undertaken. In part, this was done by testing existing *Taar1* KO mice, along with their WT littermates, for MA drinking, and for sensitivity to physiological effects and aversive effects of MA, found to be correlated with differences in MA intake in the MADR lines.

The *Taar1* transgenic mice were obtained from the U.C. Davis Knockout Mouse Project. The *Taar1* single gene KO mice were developed by electroporating or microinjecting VelociGene's Null Allele Bac vector, in which the entire *Taar1* coding region was deleted, into C57BL/6N mouse ES cells (KOMP; www.komp.org), where the mutated *Taar1* was incorporated into ES cell DNA by homologous recombination. BALB/cJ mouse embryonic blastocysts containing the *Taar1* KO ES cells are then implanted into pseudopregnant female mice (technique reviewed in Crawley, 2007). The chimeric offspring were bred with B6

mice and their offspring genotyped for the WT or KO *Taar1* segment. In order to maintain the highest level of genetic and environmental similarity between mice of the *Taar1* +/+, heterozygous (+/-), and -/- genotypes, male and female siblings, heterozygous for the targeted locus, were used to produce littermates of all three genotypes. Offspring were genotyped for *Taar1* and tested for behavioral effects of the KO, as described below.

Experimental Goals and Hypotheses

The MALDR and MAHDR mice differ in MA intake. Existing data collected in these mice indicate that factors mediating MA-induced aversion may significantly limit MA intake, but such factors have not been as well studied as those underlying drug-induced reward. The MADR lines are a genetic tool that models not only high and low MA intake, but also differential sensitivity to rewarding and aversive effects of MA (Eastwood *et al*, 2014; Eastwood and Phillips, 2012, 2014; Shabani *et al*, 2012a; b; 2011; Wheeler *et al*, 2009). In addition to the availability of these mice during the time I conducted my dissertation work, the ability to measure both positive and negative responses to MA provided a rationale for using the MADR lines of mice for this project.

The focus of this dissertation is on mechanisms that may mediate sensitivity to aversive effects of MA in the MADR lines. Because basic physiological responses to MA have not yet been characterized in these mouse lines and could play a role in subjective response to MA, two traits were chosen for examination, stress axis response and thermal response. In addition, based on existing data for the importance of TAAR1 in MA effects and the location of

Taar1 on mouse chromosome 10, within the confidence interval of the MA consumption QTL for the MADR lines, the involvement of TAAR1 in aversive response to MA, as well as MA intake, was examined.

In Chapter 2, I present frequency data from MADR mice for the *Taar1* polymorphism found in D2 and B6 mice. I hypothesized that voluntary MA consumption would be increased by the lack of functional TAAR1, and tested this by measuring MA intake in *Taar1* -/- and +/+ littermates. Additionally, I hypothesized that functional TAAR1 increases sensitivity to conditioned aversive effects of MA, and MA-induced hypothermia. As described above, MA-induced hyperthermia (Bowyer *et al*, 1994; Sabol *et al*, 2013), and hypothermia (Sabol *et al*, 2013), can occur in response to acute and chronic MA administration. MA-induced hypothermia may be partly regulated by TAAR1, based on hypothermia induced by other TAAR1 agonists, and other evidence linking hypothermic effects of MA-like drugs to TAAR1 (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010). To test this hypothesis, thermal response to MA was examined in MADR and *Taar1* transgenic mice. Thermal response to ethanol was also examined to determine drug specificity of *Taar1* influence. Here, I hypothesized that TAAR1 function does not correspond with ethanol-induced hypothermia, and thus, *Taar1* and MADR genotypes would not differ in hypothermic response to ethanol. Finally, TAAR1-related 3'-5'-cyclic adenosine monophosphate (cAMP) response to MA in B6- and D2-like TAAR1 isoforms was examined to determine functionality of the receptor.

Chapter 3 presents data for a second physiological effect of MA, activation

of the HPA axis. Given previous reports of TAAR1 agonists increasing stress response (Klieverik *et al*, 2009) and that MAHDR mice express non-functional TAAR1 (Chapter 2), I hypothesized that acute administration of MA would increase plasma stress hormone levels in MALDR and *Taar1* +/+ or +/- mice, but not MAHDR or *Taar1* -/- mice. I also hypothesized that increasing stress axis activity at the time that MA intake is being established, which would be expected to occur in MALDR mice, would reduce MA intake in MAHDR mice.

NE and 5-HT activity have been implicated in some aversive and physiological responses to psychostimulants (Jones *et al*, 2009; Jones *et al*, 2010; Rothman *et al*, 2001; Serafine and Riley, 2009), and activity of these transmitters is involved in initiation and maintenance of HPA axis response (Itoi *et al*, 1994; Makino *et al*, 2002). Given that MAHDR mice have greater expression of NET and SERT in the NAcc and that MALDR mice are more sensitive to MA-induced CTA, I hypothesized that MALDR lines would be more sensitive to the aversive effects of acute NET and SERT blockade, than MAHDR mice. I also hypothesized that repeated blockade of NET and SERT would reduce MA-induced CTA in MALDR mice through adaptations of the transporter system.

CHAPTER 2:

Trace Amine-Associated Receptor 1 Regulation of Methamphetamine Intake and Related Traits

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Abstract

Continued methamphetamine (MA) use is dependent on a positive MA experience and is likely attenuated by sensitivity to the aversive effects of MA. Bidirectional selective breeding of mice for high (MAHDR) or low (MALDR) voluntary consumption of MA demonstrates a genetic influence on MA intake. Quantitative trait locus (QTL) mapping identified a QTL on mouse chromosome 10 that accounts for greater than 50% of the genetically-determined differences in MA intake in the MAHDR and MALDR lines. The trace amine-associated receptor 1 gene (*Taar1*) is within the confidence interval of the QTL and encodes a receptor (TAAR1) that modulates monoamine neurotransmission and at which MA serves as an agonist. We demonstrate the existence of a non-functional allele of *Taar1* in the DBA/2J mouse strain, one of the founder strains of the selected lines, and show that this non-functional allele co-segregates with high MA drinking and with reduced sensitivity to MA-induced conditioned taste aversion (CTA) and hypothermia. The functional *Taar1* allele, derived from the other founder strain, C57BL/6J, segregates with low MA drinking and heightened sensitivity to MA-induced CTA and hypothermia. A role for TAAR1 in these phenotypes is corroborated in *Taar1* transgenic mice: *Taar1* knockout mice consume more MA and exhibit insensitivity to MA-induced CTA and hypothermia, compared to *Taar1* wildtype mice. These are the first data to show that voluntary MA consumption is, in part, regulated by TAAR1 function. Behavioral and physiological studies indicate that TAAR1 increases sensitivity to aversive effects of MA, and may thereby protect against MA use.

Introduction

Functional brain circuitry flexibly encodes and responds to rewarding and aversive motivational states and events. Methamphetamine (MA) is highly addictive, but has both rewarding and aversive effects that influence use. Prevention and treatment rely on knowledge of the neural mechanisms that contribute to risk for addiction and to sensitivity to the motivational effects of MA. Replicated sets of mouse lines, bidirectionally selectively bred for high or low MA drinking (MADR), are of particular relevance to the study of genetic risk for human MA use. First, in this genetic model, MA intake is completely voluntary. Second, MA high drinking (MAHDR) mice show increased sensitivity to MA reinforcement in operant intracranial and oral self-administration procedures, whereas MA low drinking (MALDR) mice do not. Third, compared to MALDR mice, MAHDR mice have greater sensitivity to conditioned rewarding effects of MA that are relevant to relapse. Finally, MAHDR mice show little sensitivity to aversive effects of MA in conditioned place and conditioned taste aversion (CTA) assays, whereas MALDR mice exhibit high sensitivity. The genetically-determined, robust sensitivity to aversive effects in MALDR mice likely limits their MA intake (Shabani *et al*, 2012a; b; 2011; Wheeler *et al*, 2009).

In addition to its well-known action as a substrate for neurotransmitter and vesicular monoamine transporters (Fleckenstein *et al*, 2007), MA is an agonist at trace amine-associated receptor 1 (TAAR1) (Bunzow *et al*, 2001; Wolinsky *et al*, 2007). Activation of TAAR1 appears to counteract some effects of MA. For example, pretreatment with the TAAR1 agonist, RO5263397, reduces operant

self-administration of MA in rats (Jing *et al*, 2015). Trace amines, such as *p*-tyramine, β-phenylethylamine, octopamine, and tryptamine, interact with this G-protein coupled receptor (Borowsky *et al*, 2001; Bunzow *et al*, 2001; Lindemann *et al*, 2005; Wolinsky *et al*, 2007), and TAAR1 modulates monoamine activity, in part, through regulation of neurotransmitter availability and disposition (Revel *et al*, 2011; Xie and Miller, 2008). TAAR1 agonists reduce endogenous firing of dopaminergic (DA), noradrenergic (NE), and serotonergic (5-HT) neurons, and *Taar1* knockout (-/-) mice exhibit greater amphetamine-induced release of these neurotransmitters in the striatum, compared to wildtype (+/+) littermates (Lindemann *et al*, 2008; Wolinsky *et al*, 2007). *Taar1* -/- mice also display greater locomotor stimulation to amphetamine and MA (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007), consistent with the idea that TAAR1 function is important for counteracting some MA effects. However, the role of TAAR1 in sensitivity to aversive effects of MA has not been examined.

Physiological effects of MA contribute to subjective effects that could impact MA consumption. Acute and chronic MA can induce hyperthermia (Bowyer *et al*, 1994; Sabol *et al*, 2013), but hypothermia may occur at lower doses and 18-20^{o} C ambient temperatures (Sabol *et al*, 2013). MA-induced hypothermia may be partly regulated by TAAR1, since other TAAR1 agonists induce hypothermia (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010), and *Taar1* -/- mice do not exhibit hypothermia to doses of the MA-like drug, MDMA, that induce hypothermia in +/+ mice (Di Cara *et al*, 2011). Likewise, *Taar1* -/- mice do not display hypothermia to MA, but the dose of MA used in this

published study did not induce hypothermia in +/+ mice (Panas *et al*, 2010), so further study is needed.

Taar1 is within the confidence interval for a quantitative trait locus (QTL) on mouse chromosome 10 (Fig. 2.1) that accounts for greater than 50% of the genetic variance in MA intake in MADR mice (Belknap *et al*, 2013). DBA/2J (D2) mice possess a unique non-synonymous single nucleotide polymorphism (SNP, C229A) in *Taar1* (Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011) that is not present in C57BL/6J (B6) mice. These are the two progenitor strains of the MADR lines. This polymorphism supports investigation of *Taar1* as a viable candidate for a quantitative trait gene (QTG) regulating MA intake.

Here, we present frequency data in MADR mice for the *Taar1* polymorphism found in D2 and B6 mice. We test the hypothesis that voluntary MA consumption is influenced by *Taar1* by measuring MA intake in *Taar1* -/-, heterozygous (+/-), and +/+ littermates. We also test the hypothesis that *Taar1* plays a role in sensitivity to a conditioned aversive effect of MA and in the thermal response to MA. In addition, we examine thermal response to ethanol to determine drug specificity of *Taar1* influence. Finally, we examine TAAR1-related 3'-5'-cyclic adenosine monophosphate (cAMP) response to MA in B6- and D2-like TAAR1 isoforms.

These studies provide the first evidence that D2 mice possess a *Taar1* allele that codes for a non-functional TAAR1, and this allele occurs at high frequency in mice that were bred for higher levels of voluntary MA consumption. Absent TAAR1 function, as found in D2, *Taar1* -/-, and MAHDR mice, increases

risk for MA consumption and decreases sensitivity to the conditioned aversive and hypothermic effects of MA. These, and published, data suggest that functional TAAR1 heightens sensitivity to certain aversive and physiological effects of MA that may limit MA use.

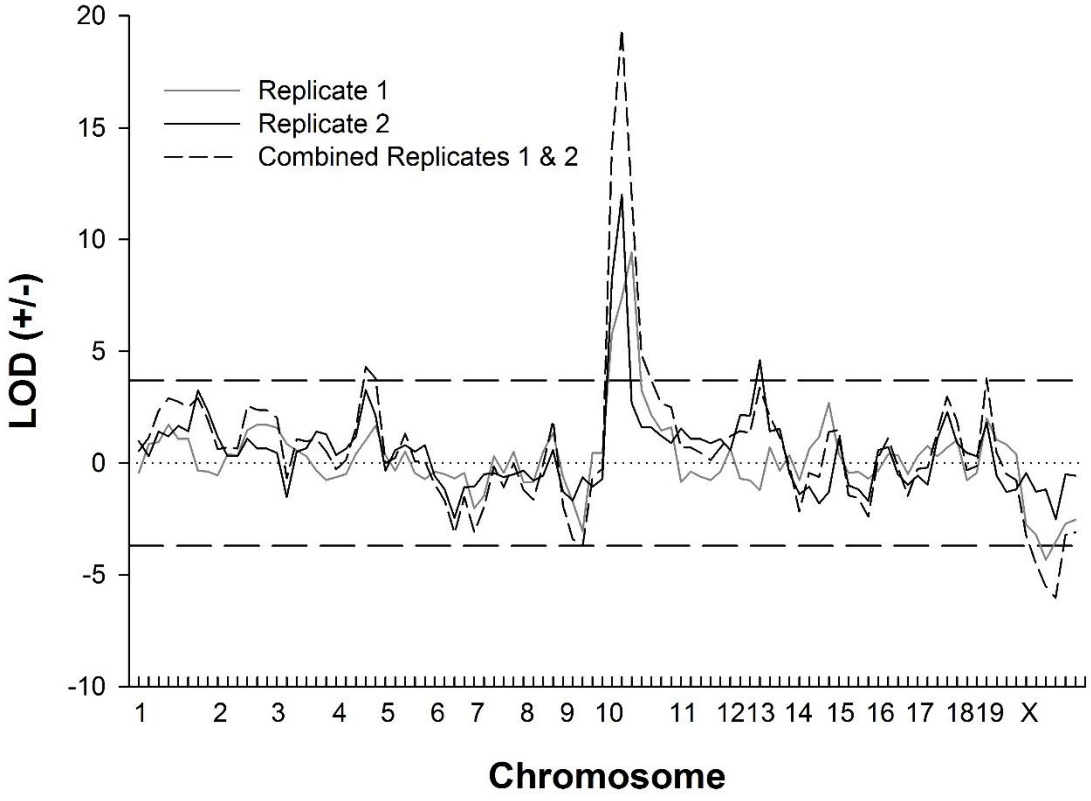

Figure 2.1. A quantitative trait locus (QTL) on mouse chromosome 10 has a major effect on MA drinking in MADR mice. The QTL on proximal chromosome 10 (10-40 Mb) explains greater than 50% of the genetic variance in the MA drinking phenotype (Belknap *et al*, 2013). Data are presented as directional genome-wide logarithm of the odds (LOD) scores. Positive and negative LOD scores indicate that higher trait scores are conferred by the D2 and B6 alleles, respectively. Dashed horizontal lines indicate statistically significant support for a QTL at $p<2 \times 10^{-5}$. Although there are a large number of genes in this interval that have the potential to influence the MA drinking trait, of particular interest based on literature supporting involvement of opioid, glutamate (GLU) and TAAR1 in MA responses, are the mu opioid receptor gene (*Oprm1*), metabotropic glutamate receptor gene (*mGlur1*), and *Taar1*, which reside at 6.76,

10.7 and 23.9 Mb, respectively. Data shown were generated using two independent sets of replicated MADR lines, produced 2 years apart. Also shown are combined data for the independent replications of the QTL study. Figure adapted from Belknap *et al* (2013), with permission.

Materials and Methods

Animals

Methamphetamine drinking selected mouse lines

The MADR mice were selectively bred from an F2 cross of the B6 and D2 inbred strains. Details of the selective breeding procedures and response to selection have been fully described (Shabani *et al*, 2011; Wheeler *et al*, 2009). Selective breeding was based on amount of the 40 mg/l MA solution consumed in the drinking procedure described below. Three consecutive pairs of MAHDR and MALDR lines have been created, with comparable outcomes, using these procedures.

Taar1 -/- Mouse Breeding and Genotyping

The *Taar1* -/- mice were obtained from the U.C. Davis Knockout Mouse Project (KOMP; www.komp.org). Briefly, chimeric mice were created by injecting BALB/c blastocysts with C57BL/6N ES cells in which the entire *Taar1* coding region was deleted by homologous recombination using VelociGene's Null Allele Bac vector. The chimeras were bred with wildtype B6 mice and their offspring genotyped according to the strategy recommended by KOMP using the following primers:

ACTCTTCACCAAGAATGTGG (forward); CCAACAGCGCTCAACAGTTC (reverse, wild type allele); GTCGTCCTAGCTTCCTCACTG (reverse, null allele). Male and female siblings, heterozygous for the targeted locus, were subsequently bred to produce *Taar1* +/+, +/-, and -/- littermates.

Prior to experiment initiation, mice were group-housed in acrylic plastic shoe-box cages (28cm × 18cm × 13cm; l × w × h), fitted with wire tops. Cages were lined with Bed-O-Cob™ (The Andersons Inc., Maumee, OH, USA) or ECOFresh™ bedding (Absorption Corporation, Ferndale, WA). Mice had free access to rodent chow (Purina 5001, 4.5% fat content; Animal Specialties Inc., Hubbard, OR) and water at all times except during testing. Colony room temperature was 20–22°C, and lights were maintained on a 12:12 h light:dark schedule, with lights on at 0600 h. Mice of both sexes were used in all studies. Procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the Veterans Affairs Portland Health Care System Institutional Animal Care and Use Committee.

Drugs and reagents

(+)MA hydrochloride was purchased from Sigma (St Louis, MO, USA) and mixed in tap water for consumption or dissolved in sterile physiological saline (0.9% NaCl; Baxter Healthcare Corporation, Deerfield, IL, USA) for injection. All injections were given intraperitoneally (i.p.) at a volume of 10 ml/kg. N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide (EPPTB) (Liu *et al*, 2014) was first diluted in DMSO, and subsequently diluted into cAMP assay buffer for a final DMSO concentration of 0.1%.

General Procedures

Taar1 sequencing

Genomic DNA from MALDR and MAHDR mice was extracted from ear or tail tissue using QuickExtract DNA Extraction Solution (Epicentre, Madison, WI). *Taar1* DNA was amplified using a Hotstart DNA polymerase kit (Qiagen, Valencia, CA) with sequence specific primers surrounding the single nucleotide polymorphism (SNP)-containing region (forward 5'-CACCAACTGGCTCCTTCACT-3', reverse 5'-CGGTGCTGGTGTGAACTTTA-3'). PCR products were run on a 1.5% agarose gel, and then purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified DNA was sequenced at the Oregon Health & Science University sequencing core using the forward primer to amplify the *Taar1* gene. Sequences of PCR products were aligned and compared with mouse *Taar1* sequence (NM_053205.1).

MA two-bottle choice drinking

Procedures were identical to those used during selection of the MADR lines (Shabani *et al*, 2011; Wheeler *et al*, 2009). MADR or *Taar1* transgenic mice were isolate housed and habituated for 48 h to drinking from two water-filled drinking tubes. During days 3-10, water was offered *vs*. 20 mg/l MA in water for 4 days, and then 40 mg/l MA in water for 4 days. Mice had 24-h access to water, but only 18-h access to MA. The positions of the water and MA tubes were alternated every 2 days to control for side preferences. Body weight was measured every 2 days and used with volume change to calculate MA consumption in mg/kg. Data from the second and fourth days for each MA

concentration (the second day after a change in MA tube position) were averaged to provide a measure of intake.

MA-induced CTA in Taar1 transgenic mice

Identical procedures for MA-induced CTA have been used to test MADR line mice (Shabani *et al*, 2012b; Wheeler *et al*, 2009). Briefly, *Taar1* +/+, +/-, and -/- mice were isolate-housed and then acclimated to water restriction (2 h of water per day) on days 1-4. On day 5, mice were introduced to the novel taste of a 0.2M NaCl solution, during a 1-h access period. Conditioning trials then occurred every other day, during which mice were given access for 1 h to the NaCl solution (days 7, 9, 11, 13, and 15), and were then immediately injected with saline or MA (2 mg/kg). This dose of MA was chosen because it induced strong CTA in MALDR mice, but no CTA in MAHDR mice. Water was made available for 30 minutes, 3 h following injections, to avoid dehydration. On intervening non-conditioning days, water was available for 2 h and no injection was administered.

MA-induced changes in body temperature

MADR or *Taar1* transgenic mice were placed in acrylic plastic chambers that isolated the mice from each other and prevented huddling-associated alterations of body temperature (1989; Crabbe *et al*, 1987). They were allowed to acclimate for 1 h and then baseline (time 0) rectal temperature was measured (Crabbe *et al*, 1987). Mice then received an injection of saline or MA (1, 2, 4, 8, or 16 mg/kg for MADR mice; 2 mg/kg for *Taar1* mice, based on dose-response results) and were returned to the chambers. Temperatures were recorded at 30,

60, 90, 120, and 180 minutes after injection. A separate set of MADR mice and *Taar1* transgenic mice were identically tested for response to ethanol (with the final reading at 300 minutes after injection) to examine whether differences in hypothermic response were specific to MA. Doses of ethanol (2 or 4 g/kg) known to induce hypothermia in mice were used (Crabbe *et al*, 1979).

Cell culture and stable transfection

HEK-293 cells were cultured as we have previously described (Eshleman *et al*, 1999; 2013). The full length coding region of the mouse *Taar1* (c-terminus GFP tag) cDNA (OriGene, Rockville, MD) was sequenced to verify that it was consistent with the B6 reference. Plasmid DNA was prepared using the Qiagen miniprep kit (Chatwsorth, CA) and Charge Switch Plasmid maxiprep kit (Invitrogen, Grand Island, NY) after transformation of BL-21 competent *E. coli* cells (Invitrogen). Sequence was verified by EcoR I/Xho I restriction enzyme digestion. The mutation at position 229 in the mouse *Taar1* gene (D2-like *Taar1*) was created using the QuickChange Lightning Kit (Agilent Technologies, Santa Clara, CA) and the B6 sequence. The mutation was verified by sequencing using the VP1.5 primer (5'-GGACTTTCCAAAATGTCG-3', OriGene). The B6- and D2-like *Taar1* expression constructs were transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen; 10 µg DNA/15cm plate) according to the manufacturer's instructions. Stably transfected cells were selected in 600 µg/ml neomycin (G418) and subsequently analyzed for cAMP accumulation in response to MA.

cAMP accumulation assay

Cells expressing the B6- and D2-like constructs were seeded at a density of 2×10^5 cells/well in 48-well tissue culture plates two days before an assay, with culture medium containing 10% FCS. One day before the assay, cells were switched to culture medium containing 10% charcoal stripped FCS and incubated overnight. Experiments were completed in assay buffer as previously described (Watts *et al*, 1998). Seven concentrations of MA (10^{-8} to 10^{-4}) were added and cells were incubated for 60 min in the presence or absence of 10 μM EPPTB (30 min pre-incubation). cAMP accumulation was measured using a cAMP EIA kit (Cayman Chemical, Ann Arbor MI), according to the manufacturer's instructions. All experiments were conducted with duplicate determinations.

Confocal microscopy

Cells containing the B6- and D2-like constructs were treated and 0.97 μm intervals were analyzed as we have previously described (Keith *et al*, 2012).

Immunodetection

Cells were lysed in RIPA lysis buffer containing 1X protease inhibitor (Roche, San Francisco, CA). Protein concentrations of the samples were determined using a BCA kit (Thermo Scientific, Waltham MA). Samples were loaded on gels using equal amounts of total protein as we have previously described (Shi and Habecker, 2012). The membrane was incubated at 4°C overnight with mouse anti-turbo GFP (1:2000, OriGene) or β-actin (1:2000, Abcam, Cambridge, MA).

Data Analysis

Behavioral and physiological data were analyzed by repeated measures analysis of variance (ANOVA), with selected line, sex, and dose as between-groups factors, and time as a within-subject factor (repeated measure). There were no interactions involving sex and thus, subsequent analyses excluded sex as a factor. Significant two-way interactions were examined using simple main effect analysis, and the Neuman–Keuls test for *post hoc* mean comparisons was applied, when appropriate. Alpha level was set at 0.05, and statistical analyses were performed using the Statistica 12 software package (StatSoft, Inc., Tulsa, OK). The MA dose-response curves for cAMP accumulation were analyzed by ANOVA, with MA dose and receptor type as between-groups factors, followed by Tukey's test for *post hoc* mean comparisons. For western blots, immunodetection analysis was carried out using LabWorks software (UVP, Upland, CA). Confocal microscope images were analyzed with LAS AF (Leica Microsystems CMS GmbH, Wetzlar, Germany). Statistical results are presented in the figure legends.

Results

Taar1 sequence

The D2 strain possesses a non-synonymous allelic variant of the *Taar1* gene (Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011), as compared to the reference B6 strain. To date, the SNP (C229A) is reported to be unique to the D2 strain; the reference B6 allele is shared by at least 27 additional strains. The SNP causes a substitution from a proline to a threonine residue at amino acid position 77 (P77T, Fig 2a), which is situated at the cytoplasmic/luminal interface of the

second transmembrane domain. We sequenced the *Taar1* gene in the MADR lines and found the D2 allele at a frequency of 1.0 in the 10 MAHDR mice sequenced; every mouse was homozygous for the D2 allele. In the 10 MALDR mice sequenced, both B6 homozygotes and B6/D2 heterozygotes were found (Fig. 2.2b). These data indicate that homozygosity for the D2 allele co-segregates with selection for high MA consumption.

MA drinking

Fig. 2.3a shows MA consumption in MADR and *Taar1* +/+, +/- and -/- mice. Shown for comparison is published MA consumption data for the progenitor B6 and D2 strains (Eastwood and Phillips, 2012). Fold changes were calculated to provide an index of magnitude of difference between genotypes. Data were collected in independent experiments and could not be legitimately included in a single statistical analysis. MAHDR mice consumed 9- and 11.9-fold more MA at the 20 and 40 mg/l concentrations (respectively), compared to MALDR mice. D2 mice consumed 3.8- and 6.6-fold more MA than B6 mice at the 20 and 40 mg/l concentrations (Eastwood and Phillips, 2012). *Taar1* +/+ and +/- mice consumed only small amounts of MA, and *Taar1* -/- mice consumed 3.3- and 6.4-fold more MA at the 20 and 40 mg/l concentrations, compared to *Taar1* +/+ mice. The dose consumed by MAHDR, D2, and *Taar1* -/- mice was significantly greater when MA was offered as a 40 mg/l concentration.

MA-induced CTA

MA-induced CTA to the novel NaCl solution was observed in *Taar1* +/+ and +/- mice, but not *Taar1* -/- mice. Statistical analyses supported significant

reductions in NaCl consumption across conditioning trials, only in the MA-treated *Taar1* +/+ and +/- mice (Fig. 2.3b). Saline treatment had no significant effect on NaCl consumption.

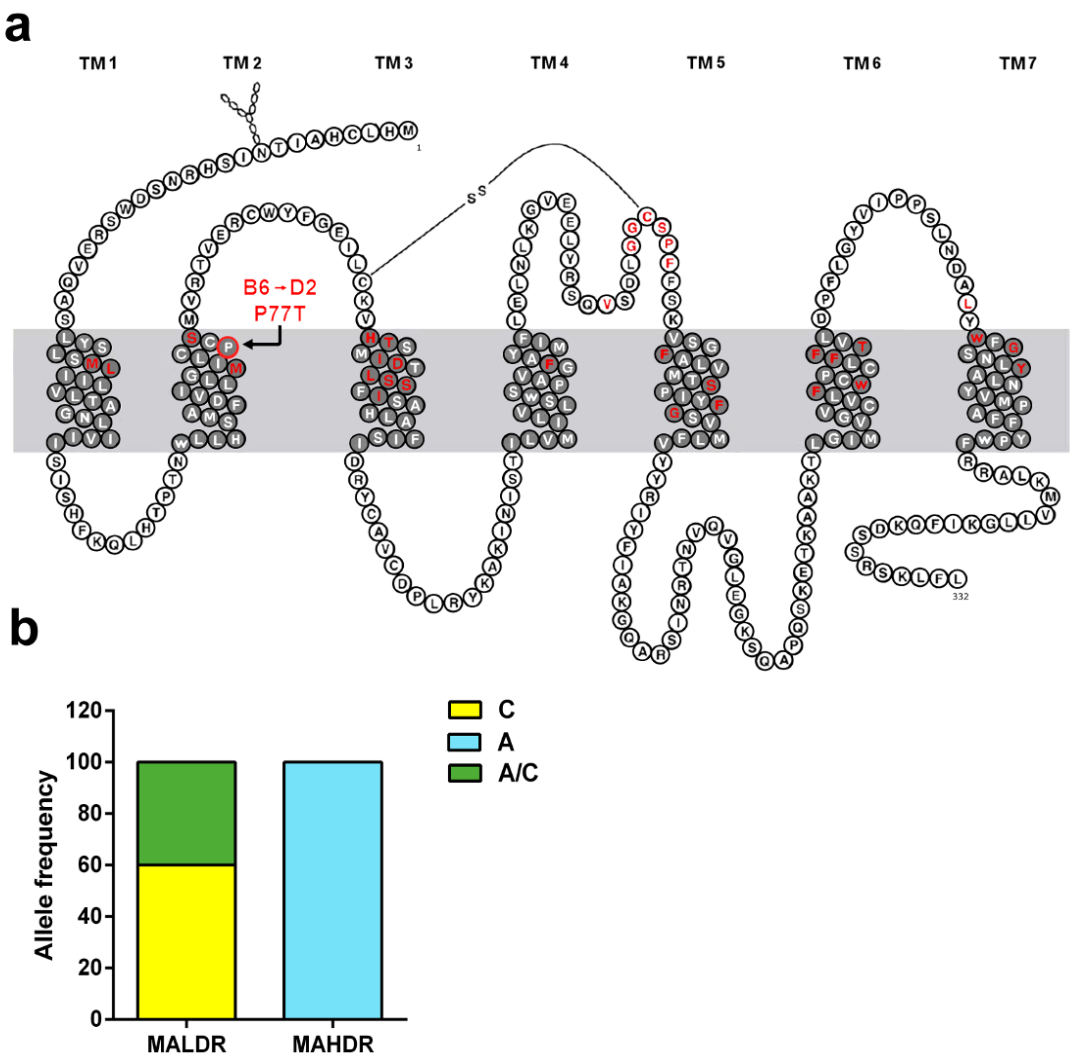

Figure 2.2. Schematic transmembrane (TM) topology of mouse TAAR1 (adapted from the human TAAR1) and frequency of B6- and D2-like *Taar1* alleles in MALDR and MAHDR mice. **(a)** Amino acid residues incorporated in the transmembrane domains are shaded in gray. Residues comprising the putative ligand binding vector in locations homologous to human TAAR1 are colored red. N-linked glycosylation at N9, as well as the disulfide bridge linking C95 and C181, are indicated according to the annotation in Uniprot entry Q923Y8_TAAR1_MOUSE. Mouse SNP rs33645709 encodes a non-synonymous

proline to threonine mutation at amino acid position 77 (P77T) in D2, compared to B6 mice. Further details are provided in the text. Figure adapted with permission from Lindemann *et al* (2005). **(b)** Frequency of B6 and D2 *Taar1* alleles in MALDR and MAHDR mice. *Taar1* was sequenced in MALDR and MAHDR mice (n=10/line; replicate 2, selection generation 5). “A” and “C” refer to adenine and cytosine, respectively. MAHDR mice are homozygous for the D2 allele at nucleotide 229. This SNP leads to a threonine at amino acid position 77. MALDR mice are either homozygous or heterozygous for the B6 allele. B6: C57BL/6J; D2: DBA/2J; MALDR: MA low drinking; MAHDR: MA high drinking.

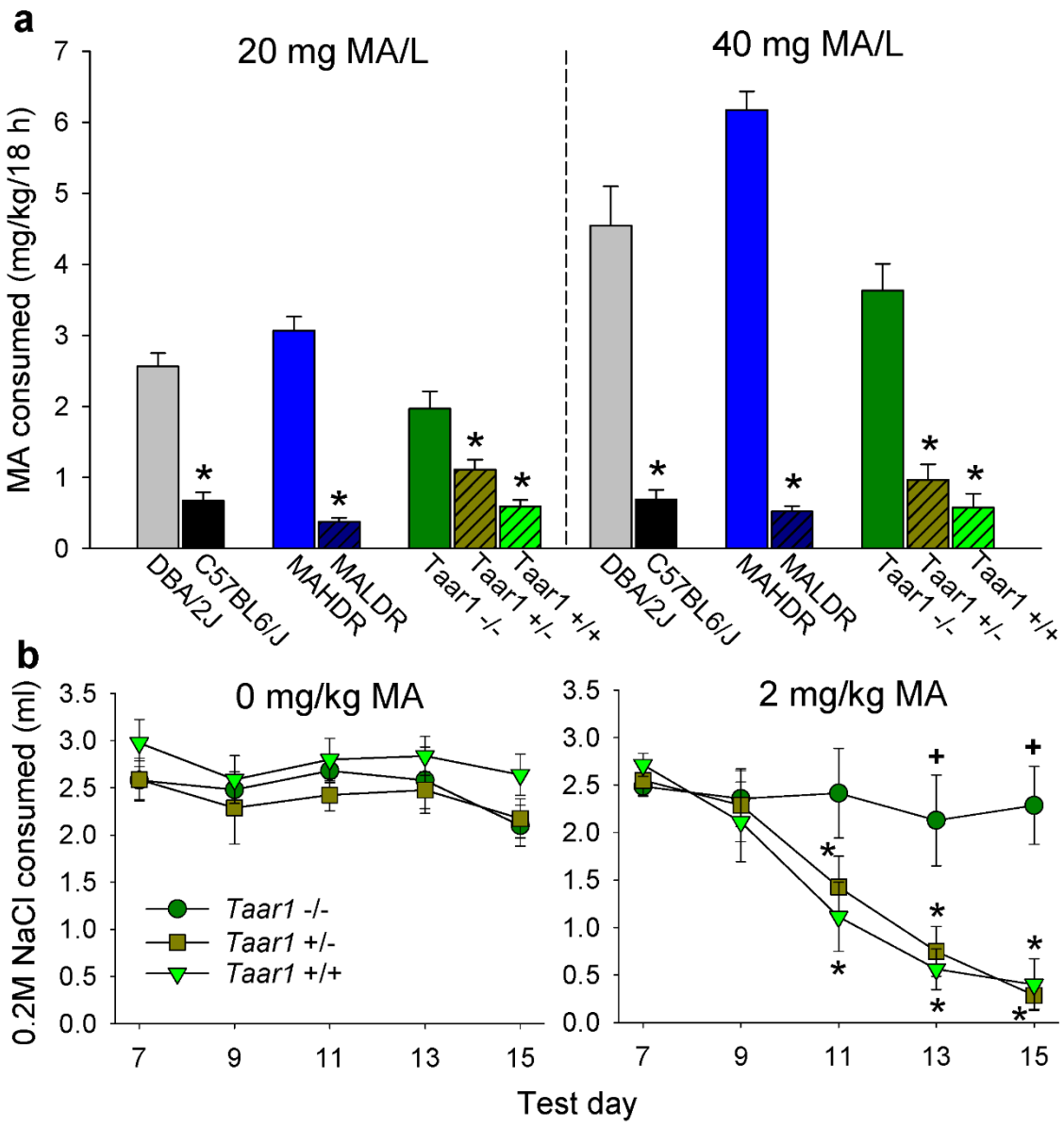

Figure 2.3. (a) Methamphetamine (MA) consumption differs by *Taar1* genotype. DBA/2J (D2) mice consume more MA in mg per kg than C57BL/6J (B6) mice, as previously reported (Eastwood *et al*, 2012). Data shown here support the greater MA consumption of MAHDR mice, compared to MALDR mice. There was a significant line x concentration interaction [$F(1,122)=121.3$; $p<0.0001$]; MA intake was higher in MAHDR mice for both MA concentrations, but the difference was

greater for 40 mg/L MA. *Taar1* -/- mice consumed more MA than *Taar1* +/- or +/+ mice. There was a genotype x concentration interaction [$F(2,63)=14.4$ $p<0.001$]; MA intake differed for both MA concentrations, but the difference was greater for 40 mg/L MA. *****: $p<0.05$ for the difference between the lines or genotypes (D2 vs. B6, MAHDR vs. MALDR, *Taar1* -/- vs. *Taar1* +/+ and *Taar1* -/- vs. *Taar1* +/-) within each concentration. N=62/MADR line (49-54 days old; replicate 3, selection generation 4), and 19-28/transgenic genotype (95-365 days old). B6 and D2 data are shown here with permission (Eastwood *et al*, 2012). MALDR: MA low drinking; MAHDR: MA high drinking. **(b)** Sensitivity to MA-induced conditioned taste aversion (CTA) differs by *Taar1* genotype. *Taar1* -/- mice were insensitive to MA-induced CTA at doses that produce CTA in *Taar1* +/+ and +/- mice. There was a significant genotype x treatment x day interaction [$F(8,152)=2.7$ $p<0.01$]. Subsequent analysis in MA-treated mice identified a significant genotype x day interaction [$F(8,80)=4.17$ $p<0.0005$] that was not found in saline-treated mice. Shown are means ± SEM. +: $p<0.05$ for the difference between *Taar1* -/- vs. *Taar1* +/+ and *Taar1* -/- vs. *Taar1* +/- on specific day; *****: $p<0.05$ for the difference in NaCl consumption on the indicated day, compared to consumption on day 7 prior to conditioning, within genotype, N=5-8/ transgenic genotype for saline, and 7-8/transgenic genotype for MA (109-176 days old).

Thermal response to MA

Fig. 2.4a shows the core body temperature response to multiple doses of MA in replicate 2 MADR mice. MAHDR mice exhibited a hyperthermic response to all doses of MA (1 to 16 mg/kg), whereas the primary response in MALDR mice was hypothermia. Similar data were generated in replicate 3 MADR mice (Fig. 2.5). *Taar1* transgenic mice were subsequently tested with 2 mg/kg MA (Fig. 2.4b), because this dose produced a clear difference in hypothermic response in both replicate sets of MADR mice. Data for MADR mice are reproduced in Fig. 2.4c from Fig. 2.4a to facilitate direct comparison. *Taar1* +/+ and +/- mice responded similarly to MALDR mice, showing hypothermia, whereas *Taar1* -/- mice did not experience significant hyper- or hypothermia. Therefore, the hypothermic response occurred in mice that possess the B6-like *Taar1* allele. The difference in sensitivity to the hypothermic effect of MA did not generalize to ethanol, as all genotypes showed hypothermia and there were no genotype-dependent differences (Fig. 2.4d,e).

TAAR1 function and MA consuming mice

To determine differences in function between the B6- and D2-like isoforms of TAAR1, site-directed mutagenesis was used to create the D2 construct found in all MAHDR mice. Both the wildtype and mutant constructs were stably transfected into HEK-293 cells, cells were treated with the TAAR1 agonist, MA, and cAMP accumulation was measured using ELISA. MA elicited a dose-dependent response in cells expressing B6-like TAAR1 (EC_{50} =826 nM), and the effect was blocked by the TAAR1 antagonist, EPPTB (Fig. 2.6). cAMP

accumulation was absent following MA treatment of non-transfected cells and of cells expressing D2-like TAAR1, suggesting that the receptor is non-functional. Immunoblot verified that both the functional B6- and the non-functional D2-like receptors were expressed in transfected HEK-293 cells. Confocal microscopic analysis of GFP-tagged constructs corroborated the immunoblot data, and indicated that both forms of TAAR1 are cytosolic, consistent with previous reports (Bunzow *et al*, 2001; Xie *et al*, 2007).

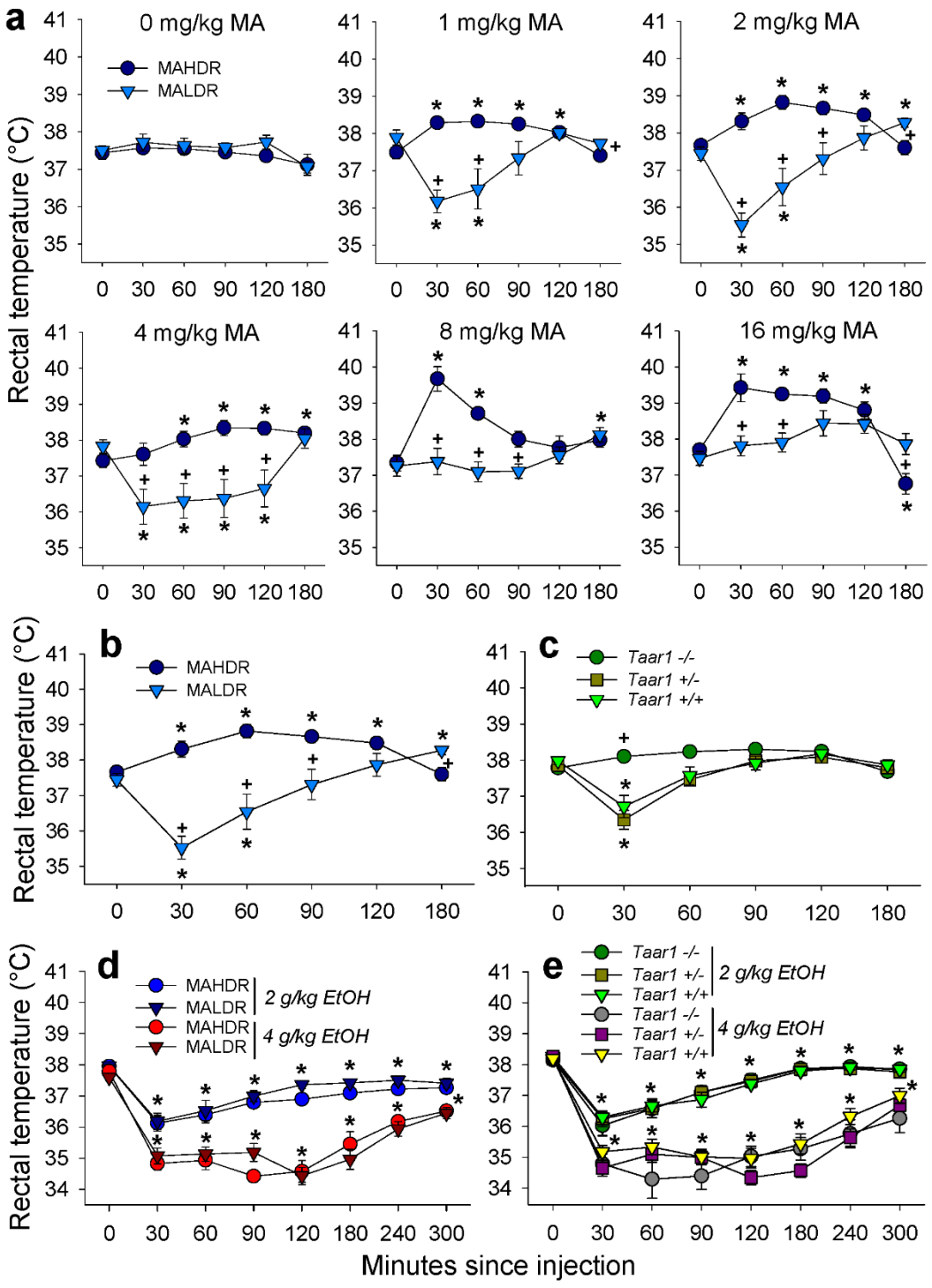

Figure 2.4. Methamphetamine (MA)-induced hypothermia differs by *Taar1* genotype. **(a)** MALDR mice exhibited hypothermia in response to 1, 2, and 4

mg/kg MA, whereas MAHDR mice exhibited hyperthermia in response to all MA doses. There was a significant line x time x dose interaction [F(25,800)=3.2; p<0.0001] and subsequent analysis identified a significant line x time interaction at each dose of MA [F's(5,105-165)=7.5-22.8; all p's<0.0001]. Significant changes in temperature across time were found in both lines of mice at all MA doses, except in MALDR mice at 16 mg/kg MA. Thermal data in saline-treated mice were similar for the MALDR and MAHDR lines, and there were no significant differences in body temperature from baseline, except for a small reduction at 180 minutes. N=12/MADR line and dose; 62-99 days old; replicate 2, selection generation 5. **(b and c)** *Taar1* +/+, and *Taar1* +/- mice exhibit hypothermia in response to 2 mg/kg MA, similar to MALDR mice, whereas the hypothermic response is absent in MAHDR and *Taar1* -/- mice. Data for 2 mg/kg MA in MADR mice are shown in **(b)** to facilitate comparison to *Taar1* transgenic mice, and are the same data shown in **(a)**. For the *Taar1* transgenic mice **(c)**, there was a significant interaction of genotype x time [F(10,80)=3.3 p<0.005] and significant changes in temperature across time were found for all transgenic genotypes, except *Taar1* -/-. N=11-13/transgenic line and dose (92-290 days old). **(d and e)** Ethanol-induced hypothermia was induced by both 2 and 4 g/kg ethanol (EtOH) in MADR mice and *Taar1* transgenic mice and no significant differences were found. **(d)** For MAHDR and MALDR mice, there was a significant time x dose interaction [F(7,329)=17.1; p<0.0001], with greater hypothermia induced by the higher ethanol dose. N=12-13/MADR line; 86-96 days old; replicate 2, selection generation 5. **(e)** For *Taar1* transgenic mice, there

was a significant time x dose interaction [F(7,581)=18.0; $p<0.0001$], with greater hypothermia induced by the higher ethanol dose. N=12-16/ transgenic line and dose (140-220 days old). Mean comparisons collapsed on genotype identified significant differences in core temperature between the baseline measure and after ethanol treatment. Note: *Taar1* mice were in short supply and prior to testing for ethanol-induced hypothermia, had been included in another study in which MA was given 5 times at a frequency of every 48 h, and then allowed a 2-week rest interval between the studies. Shown are means ± SEM. +: $p<0.05$ for the difference between the lines or genotypes; *****: $p<0.05$ for the difference between baseline temperature and temperature at a given time point within a given genotype, or in the case of ethanol, for the genotypes collapsed. MADR: MA drinking; MALDR: MA low drinking; MAHDR: MA high drinking.

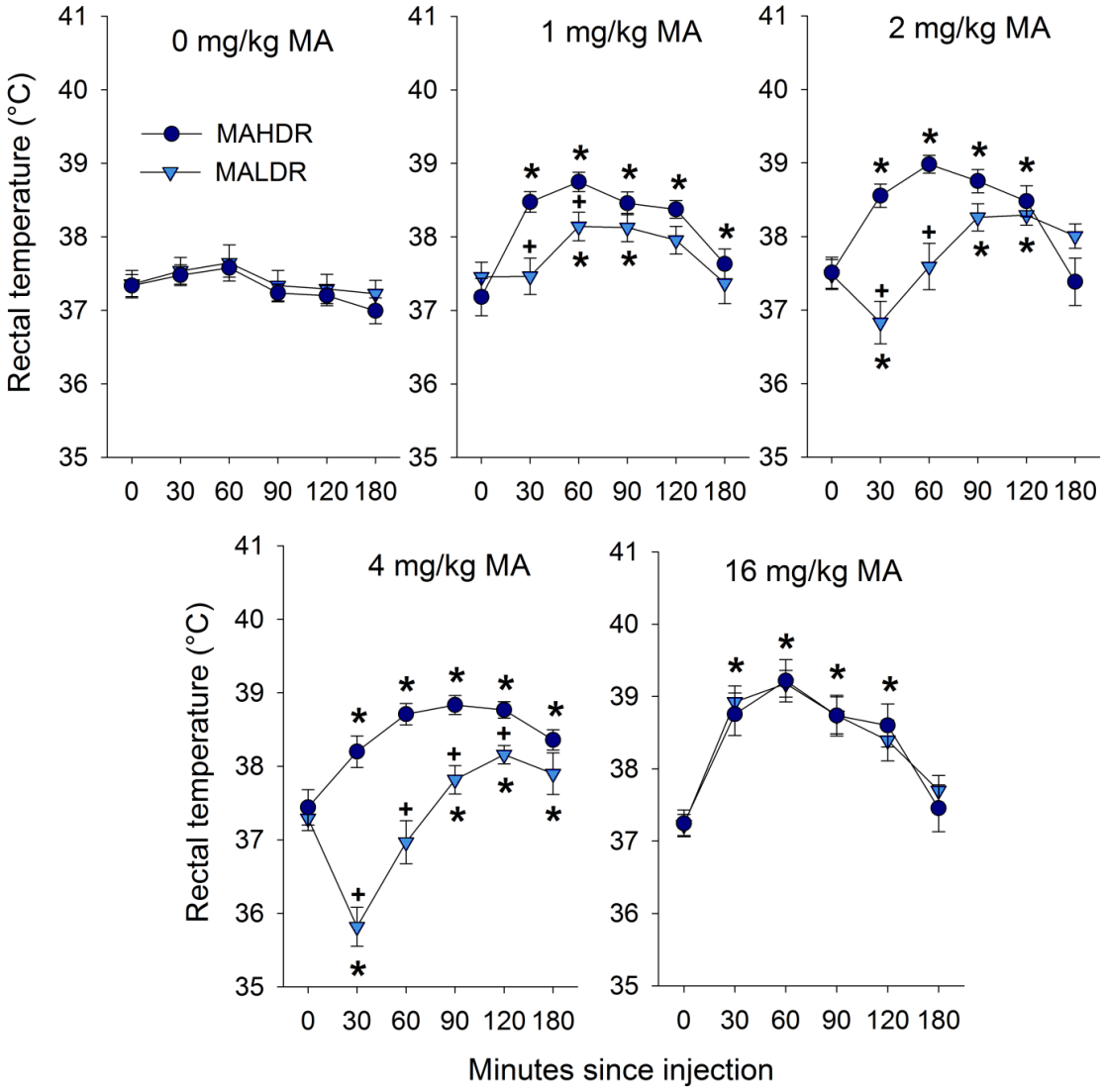

Figure 2.5. The effect of MA on body temperature differs in replicate 3 MAHDR and MALDR mice. MALDR mice exhibited hypothermia in response to 2 and 4 mg/kg MA, whereas MAHDR mice exhibited hyperthermia in response to all doses of MA. Hyperthermia in MALDR mice was less pronounced except at 16 mg/kg MA. Core body temperature was measured as described in the manuscript text. There was a significant line x time x dose interaction [$F(20,565)=4.4$; $p<0.0001$]; subsequent analysis identified a significant line x time interaction at

each dose of MA [F's(5,110-120)=4.2-16.1; all *p's*<0.005], except 16 mg/kg. Thermal data in saline-treated mice were similar for the MALDR and MAHDR lines, and there were no significant differences in body temperature from baseline. Shown are means ± SEM. +: *p*<0.05 for the difference between the lines. *: *p*<0.05 for the difference between baseline temperature and temperature at a given time point within mouse line. N=11-14/line and dose; 62-88 days old; replicate 3, selection generation 3. MA: methamphetamine; MALDR: MA low drinking; MAHDR: MA high drinking.

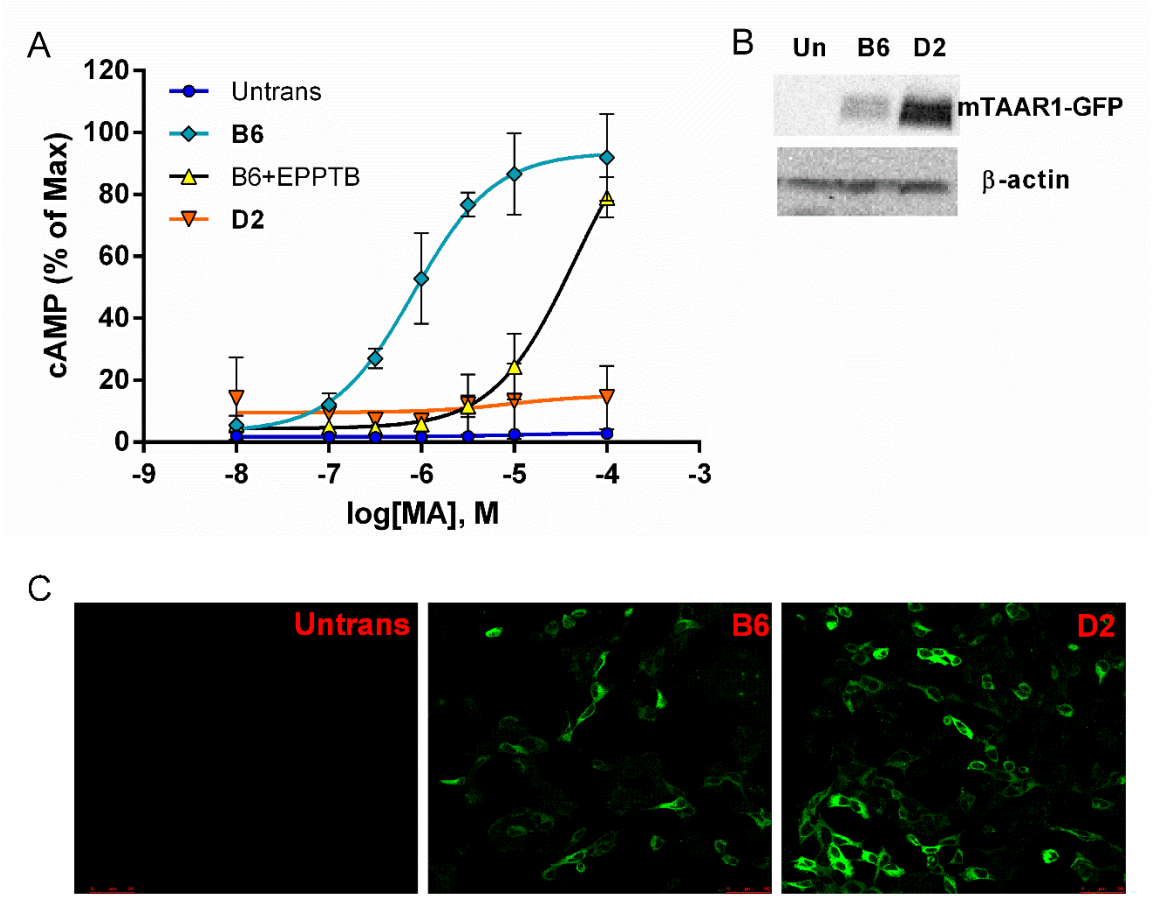

Figure 2.6: The B6-like, but not D2-like, isoform of TAAR1 is activated by MA in *Taar1*- transfected cells. HEK293 cells were stably transfected with GFP-tagged B6- or D2-like *Taar1*, and cAMP assays were performed as described in the text. **(a)** The B6-like isoform of TAAR1 responds to MA stimulation (EC_{50} = 826 nM), however, the D2-like isoform does not, suggesting that the receptor is non-functional. Administration of the TAAR1 antagonist EPPTB (EC_{50} ~ 60 µM) produced a right-ward shift in MA-induced cAMP accumulation in the B6-like recombinant TAAR1. Data shown are the average of at least 3 independent experiments, each conducted with duplicate determinations. Shown are means ± SEM. There was a significant dose x receptor type interaction [F(18,56)=23.4;

p<0.0001]. *: *p*<0.01 for the comparison between the indicated group and untransfected control. +: *p*<0.01 for the comparison between the MA alone and MA + EPPTB C57BL/6J TAAR1 groups. **(b)** Both the functional B6-like as well as the non-functional D2-like isoforms of TAAR1 were expressed in transfected cells. Untransfected (Untrans) cells did not express TAAR1. β-actin was measured as a loading control. **(c)** Confocal images demonstrating expression of the functional B6-like and non-functional D2-like TAAR1 in cells. B6: C57BL/6J; D2: DBA/2J.

Discussion

In the current experiments, MA drinking, sensitivity to MA-induced CTA and thermal response to MA corresponded with *Taar1* genotype. Homozygous expression of a non-functional isoform of TAAR1 was associated with heightened genetic risk for MA intake. Homo- or heterozygous expression of functional TAAR1 appears to protect against MA consumption, and suggests that the D2 *Taar1* polymorphism is not a dominant negative mutation. However, the functional *Taar1* allele is dominant in its effect on MA intake. The segregation of the D2-like and B6-like alleles in MAHDR and MALDR mice confirms the direction of allele influence predicted by the QTL on chromosome 10 (Belknap *et al*, 2013). Overall, these data provide strong support for *Taar1* as a candidate gene for regulation of MA consumption. However, the MA consumption phenotypes of the MAHDR and MALDR mice were more extreme than those of the non-selectively bred D2/B6 and transgenic mice, as indicated by the fold-difference data. The influence of other genes is supported by this finding and by the finding that the chromosome 10 QTL accounts for about half, not all, of the genetically determined variance in MA intake.

Lower genetic risk for MA consumption was associated with sensitivity to MA-induced CTA and hypothermia. This outcome was observed in two genetic models. Transgenic mice homozygous or heterozygous for a functional *Taar1* allele avoid MA consumption and are sensitive to MA-induced CTA and hypothermia. Similarly, MALDR mice, which are either homozygous or heterozygous for a functional *Taar1* allele, avoid MA consumption and are

sensitive to aversive effects of MA (Shabani *et al*, 2012b; 2011; Wheeler *et al*, 2009) and MA-induced hypothermia. This outcome is clear in two replicate sets of MADR lines, which strongly supports common genetic influence on MA consumption and sensitivity to the aversive and hypothermic effects of MA. Furthermore, combined with data from the *Taar1* transgenic mice, these data suggest *Taar1* as a candidate gene that influences all 3 traits. Alternatively, *Taar1* may regulate one response which influences the others. For example, *Taar1*-regulated sensitivity to MA-induced hypothermia may cause a reduction in MA consumption or underlie conditioned aversion. This hypothesis could be tested by preventing the hypothermic effect in MALDR mice while measuring MA intake. MADR mice do not differ in locomotor stimulation to 0.5, 2, or 4 mg/kg MA (Shabani *et al*, 2011); therefore, genotype-specific differences in hypothermia at these doses of MA are not likely due to differential locomotor activation by MA. However, unpublished data in the MADR lines indicate greater sensitivity of MAHDR mice, like *Taar1* -/- mice, to the locomotor stimulant effects of some higher doses of MA. Finally, sensitivity to ethanol-induced hypothermia appears to be regulated by genetic factors distinct from those that influence sensitivity to the thermal effects of MA, as the response to ethanol was similar in both the transgenic mice and MADR mice.

Risk for drug use is affected by the balance of positive and negative experiences with a drug (Cruickshank and Dyer, 2009; Davis and Riley, 2010). Considerable attention has been given to studying positive rewarding effects associated with MA addiction (Beckmann *et al*, 2010; Horton *et al*, 2011; Kamens

et al, 2005a; Mahler *et al*, 2013; Meyer *et al*, 2011; Mizoguchi *et al*, 2004; 2012a; Shabani *et al*, 2011; Wheeler *et al*, 2009), whereas aversive effects that could limit intake have been given less consideration (Harrod *et al*, 2010; Pringle *et al*, 2008; Shabani *et al*, 2012b; 2011; Wheeler *et al*, 2009). Greater sensitivity to the hyperthermic effects of MA did not correspond with reduced voluntary MA drinking. Instead, heightened sensitivity to MA-induced hypothermia was associated with low MA intake and greater sensitivity to MA-induced aversion. MA is an agonist at TAAR1 (Bunzow *et al*, 2001; Reese *et al*, 2014; Wolinsky *et al*, 2007) and the outcome of hypothermia is in agreement with other reports of TAAR1 agonist-induced hypothermia in rodents (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010; Sabol *et al*, 2013). Thus, it appears that TAAR1 mediates MA-induced hypothermia and that the immediate hypothermic effect of MA may play a role in curbing MA intake in MALDR, *Taar1* +/+ and *Taar1* +/- mice. Reduced body temperature alone does not induce CTA in rodents (Misanin *et al*, 1998). However, hypothermia did prolong the associative period during which aversion could be conditioned (Christianson *et al*, 2005; 2002; Misanin *et al*, 1998). Therefore, one possible role of the hypothermic response in MA consumption and MA-induced CTA is that MA-induced hypothermia may increase the association of MA with unpleasant physiological or subjective effects.

MA causes synaptic release of DA and other monoamines, including NE and 5-HT (Fleckenstein *et al*, 2007; Rothman *et al*, 2001). MA de-vesicularizes monoamines, which diffuse into the cytoplasm and can then be reverse

transported into the synapse (Fleckenstein *et al*, 2007). Genes encoding the NE transporter (*Slc6a2*), and the 5-HT transporter (*Slc6a4*), but not the DA transporter (*Slc6a3*), are more highly expressed in nucleus accumbens (NAcc) tissue from MAHDR mice than MALDR mice (Wheeler *et al*, 2009). These genes are not located on mouse chromosome 10, and are therefore not candidates for the QTG in that region (Belknap *et al*, 2013). However, *Taar1* is within the QTL interval, and it modulates monoamine levels by altering transporter function in mice and primates (Miller, 2011, 2012; Revel *et al*, 2011; Xie and Miller, 2008, 2009). Furthermore, *Taar1* -/- mice exhibit lower basal levels and greater amphetamine-induced release of DA in the striatum, compared to +/+ mice (Lindemann *et al*, 2008; Wolinsky *et al*, 2007). Similarly, MAHDR mice, which carry the non-functional version of the TAAR1, also exhibit lower resting DA tone in the NAcc and medial prefrontal cortex (mPFC), and higher MA-induced DA release in the mPFC, but not NAcc (Lominac *et al*, 2014). Therefore, DA-related phenotypes may be associated with level of MA intake. On the other hand, differences in 5-HT disposition in MADR and *Taar1* transgenic mice do not correspond. *Taar1* -/- mice have lower basal levels of 5-HT and greater amphetamine-induced 5-HT release compared to +/+ mice (Wolinsky *et al*, 2007), whereas the opposite relationship is seen in MADR mice; MAHDR mice have higher basal levels of 5-HT in the NAcc and show reduced sensitivity to MA-induced increases in 5-HT (Lominac *et al*, 2014). Different brain regions and assay methods could explain discrepancies related to 5-HT. On the other hand, 5-HT may not play a role in genetically-determined differences in MA intake.

Ours is the first report of voluntary MA intake in animals with genetic alterations resulting in loss of TAAR1 function. A recent publication involving a pharmacological manipulation of TAAR1 yielded similar results (Jing *et al*, 2015). The TAAR1 agonist, RO5263397, dose-dependently reduced MA self-administration in rats, just as functional TAAR1 in our studies was associated with reduced voluntary MA intake. In the study in rats, reinstatement of MA-seeking was also attenuated by the TAAR1 agonist, whereas the TAAR1 agonist had no effect on reinstatement of sucrose-seeking. MADR mice consume similar amounts of saccharin and quinine, indicating that TAAR1 function in this genetic model does not play a role in the consumption of a natural reward or bitter substance (Shabani *et al*, 2011; Wheeler *et al*, 2009). A TAAR1 agonist approach cannot be taken in our mice, because the receptor is non-functional.

The 999 bp mouse *Taar1* on chromosome 10 is phylogenetically related to the 1020 bp human *TAAR1* on chromosome 6 (Lindemann *et al*, 2005), and the 332 amino acid mouse receptor shares 76% homology with the 339 amino acid human receptor (Borowsky *et al*, 2001). There are a number of reported synonymous and non-synonymous SNPs in the human *TAAR1* (dbSNP, NCBI, 2014), but there are no reported polymorphisms that are shared across the mouse and human. Some of the reported non-synonymous SNPs in the human *TAAR1* are located in regions that should alter receptor recognition of ligand or receptor function (Pardo *et al*, 1992). Non-functional TAAR1 in mice is associated with higher levels of voluntary MA consumption and reduced sensitivity to aversive effects of MA. It is possible that the TAAR1 limits MA

consumption in some humans by conferring sensitivity to aversive effects of MA. Therefore, drugs that stimulate a sub-functional TAAR1 may increase aversive effects and be useful for treating MA addiction.

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CHAPTER 3:

Trace Amine-Associated Receptor 1 and Stress Axis Regulation of Methamphetamine Intake

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Abstract

Methamphetamine (MA) Low Drinking (MALDR) mice are sensitive to aversive, but not rewarding effects of MA. The opposite sensitivities have been found in MA High Drinking (MAHDR) mice. Existing data in these mice and null mutant mice for the trace amine-associated receptor 1 (TAAR1) suggest an association of sensitivity to these effects of MA with TAAR1. MA is a substrate for TAAR1, which in turn regulates monoamine systems. We hypothesized that MA administration would result in greater plasma corticosterone (CORT) and adrenocorticotropic hormone (ACTH) response in MALDR mice, which express functional TAAR1, than in MAHDR mice, which express a non-functional TAAR1 isoform. We also measured CORT response to MA in *Taar1* null mutant (-/-) and wildtype (+/+) mice. MA increased ACTH and CORT levels in MALDR and +/+ mice, but not in MAHDR or -/- mice. We also tested whether elevated CORT during MA drinking was sufficient to reduce MA intake. MA preference was decreased in MAHDR mice when CORT was included in the MA solution. Finally, we tested the hypothesis that NE and 5-HT transporter blockade are involved in sensitivity to the aversive effects of MA. Low-dose fluoxetine induced CTA more quickly in MALDR than MAHDR mice, and prior treatment with fluoxetine slowed the development of MA-induced CTA in MALDR mice. TAAR1 appears to mediate differences in stress axis response to MA, and SERT function may underlie some of the differential sensitivity to aversive effects of MA in the MA drinking selected lines.

Introduction

Not including alcohol and nicotine, methamphetamine (MA) is the second most abused psychoactive drug worldwide, behind only marijuana in number of users, and exceeding the use of cocaine and heroin combined (WHO, 2014). MA has significant detrimental health effects when used chronically (Volkow, 2013), but not all initial users develop chronic use patterns. Whether an individual continues to use MA is likely affected by the balance of positive and negative subjective effects of the drug (Cruickshank and Dyer, 2009; Davis and Riley, 2010). Previous research has focused heavily on mechanisms of action that determine the consequences of acute and chronic MA effects, and on rewarding effects of MA that may predict the likelihood of continued use (e.g. Beckmann *et al*, 2010; Bryant *et al*, 2009a; 2009b; Horton *et al*, 2011; Ikeda *et al*, 2007; Kamens *et al*, 2005b; Kelly *et al*, 2008; Meyer *et al*, 2011; Mizoguchi *et al*, 2004; Palmer *et al*, 2005; Shabani *et al*, 2012a; 2011). Sensitivity to the aversive effects of MA has not been given the same level of consideration (e.g. Pringle *et al*, 2008; Rothman *et al*, 2001; Shabani *et al*, 2012b; 2011). We have completed the production of three replicate sets of mouse lines that were bidirectionally selectively bred for amount of MA consumed (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009). The high and low MA drinking (MADR) lines were selectively bred on the basis of voluntary MA intake in a two-bottle choice procedure, but they also reliably differ in sensitivity to rewarding and aversive effects of MA (Harkness *et al*, 2015; Shabani *et al*, 2012b; 2011; Wheeler *et al*, 2009). Furthermore, the MA high drinking (MAHDR) line exhibits operant

behavior consistent with reinforcement by MA, whereas the MA low drinking (MALDR) line does not (Shabani *et al*, 2012a). Therefore, this genetic animal model provides a vehicle for investigation of genetic influences and mechanisms associated not only with MA intake, but also reinforcing, rewarding and aversive effects of MA.

MA is a substrate for synaptic and vesicular monoamine transporters (Bunzow *et al*, 2001; Fleckenstein *et al*, 2007; Wolinsky *et al*, 2007). Recently, the trace amine-associated receptor 1 (TAAR1) has garnered considerable attention because MA, along with trace amines such as *p*-tyramine, *β*-phenylethylamine, octopamine, and tryptamine, have agonist effects at this receptor (Bunzow *et al*, 2001; Wolinsky *et al*, 2007). The encoding gene, *Taar1*, is located at 23.96 Mb on mouse chromosome 10, within the confidence interval of a quantitative trait locus (QTL) that accounts for approximately 50% of the genetically attributable variation in MA intake (Belknap *et al*, 2013). Low MA intake in MALDR mice, C57BL/6J mice (one of the progenitor strains of the MADR lines) and *Taar1* wildtype (+/+) mice is inversely associated with TAAR1 function (Harkness *et al*, 2015). MAHDR and DBA/2J mice (the other MADR progenitor strain) express a non-functional TAAR1 isoform (Harkness *et al*, 2015) resulting from a non-synonymous single nucleotide polymorphism (SNP, C229A) in *Taar1* (Sanger Mouse Genome Project SNP Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011). MAHDR, DBA/2J, and *Taar1* null mutant (-/-) mice consume larger amounts of MA. Furthermore, mice expressing functional TAAR1 are sensitive to MA-induced conditioned taste aversion (CTA) and become

hypothermic when administered lower doses of MA (1-4 mg/kg), whereas these effects are not seen in mice without functional TAAR1. Thus, sensitivity to certain physiological and aversive effects of MA may discourage MA consumption. Although TAAR1 appears to mediate sensitivity to these MA-related effects (Harkness *et al*, 2015), the precise mechanism(s) through which this occurs has not been fully determined.

In addition to hypothermia, hypothalamic-pituitary-adrenal (HPA) axis (stress axis) activation may be another outcome of MA exposure that is related to MA-induced aversion. HPA activation involves increases in corticotropin-releasing factor (CRF) followed by adrenocorticotropin hormone (ACTH) release from the anterior pituitary, and corticosterone (CORT) release from the adrenal cortex. Increases in plasma ACTH and CORT levels have been measured after acute treatment with MA (Grace *et al*, 2008; Szumlinski *et al*, 2001; Williams *et al*, 2000). We hypothesized that MA-induced stress axis effects are greater in the MALDR than the MAHDR line, a physiological outcome that could partially explain greater MA aversion in the MALDR line. Additionally, increases in plasma CORT levels have been reported in rats following activation of TAAR1 by the agonists, T_1AM and T_0AM (Klieverik *et al*, 2009). Thus, non-functional TAAR1, as found in MAHDR mice, could result in a reduced stress axis response to MA and may be relevant to the reduced sensitivity to the aversive effects of MA in MAHDR mice.

TAAR1 is a G-protein coupled receptor that modulates monoamine activity through regulation of neurotransmitter availability and disposition (Revel *et al*,

2011; Xie and Miller, 2008). Agonist binding reduces firing of dopaminergic (DA), noradrenergic (NE), and serotonergic (5-HT) neurons (Lindemann *et al*, 2008; Revel *et al*, 2011; Wolinsky *et al*, 2007), and the TAAR1 agonist, RO5263397, reduces MA self-administration in rats (Jing *et al*, 2015). Knockout mice lacking functional TAAR1 have heightened sensitivity to amphetamine and MA locomotor stimulation and greater release of DA, 5-HT, and NE in the striatum after amphetamine administration, compared to wildtype mice (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007). MAHDR mice lack functional TAAR1 and have lower resting dopaminergic tone in the nucleus accumbens and medial prefrontal cortex (mPFC), but higher dopamine release following MA in the mPFC (Lominac *et al*, 2014). Altered regulation and MA-induced release of monoamines in mice lacking functional TAAR1 may contribute to high sensitivity to the aversive effects of MA and protect against voluntary MA intake.

MA-naïve MAHDR mice have higher expression of the genes encoding the NE transporter (NET; *Slc6a2*) and 5-HT transporter (SERT; *Slc6a4*) in NAcc tissue (Wheeler *et al*, 2009). This may result in more efficient removal of these monoamines from the synapse. NE and 5-HT activity have been implicated in some aversive and physiological responses to MA and cocaine, another psychostimulant that blocks monoamine transporters (Hassan *et al*, 2015; Jones *et al*, 2009; Rothman *et al*, 2001; Serafine and Riley, 2009). Additionally, activity of these transmitters is involved in initiation and maintenance of HPA axis response to stress (Itoi *et al*, 1994; Makino *et al*, 2002). These findings, combined with the role of TAAR1 in regulation of monoamine activity, and

differences in TAAR1 function between the MADR lines, supports testing NET and SERT blockers for their ability to mimic and manipulate the aversive response to MA in MADR mice.

Here, we tested whether acute administration of MA would differentially stimulate plasma ACTH and CORT levels in MAHDR, MALDR, *Taar1* null mutant, and wildtype mice, predicting that mice sensitive to MA-induced CTA would also experience increased MA induction of stress hormone levels. We also tested the hypothesis that increasing stress axis activity at the time that MA intake is being established, which would be expected to occur in MALDR mice during the first drinking session, would reduce MA intake in MAHDR mice. Lastly, we tested the hypothesis that MAHDR and MALDR lines are differentially sensitivity to the aversive effects of acute NET and SERT blockade using nisoxetine (NISX) and fluoxetine (FLUX), respectively. We hypothesized that repeated blockade of NET and SERT would lead to reduced CTA to MA through adaptations of the transporter system. We provide the first evidence that MA-induced elevated plasma CORT levels correspond with TAAR1 function and level of MA intake, and that CORT administered in drinking fluid can reduce MA intake in mice. Additionally, we report that blockade of SERT may be one mechanism through which aversive effects of MA are mediated. These and published data suggest that sensitivity to aversive and physiological effects of MA are closely linked to TAAR1 function, which may in turn limit MA use.

Methods

Animals

All procedures were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the VA Portland Health Care System (VAPORHCS) Institutional Animal Care and Use Committee. Prior to experiment initiation, mice were housed (1-5 per cage) in acrylic plastic shoe-box cages (28cm × 18cm × 13cm; l × w × h) fitted with wire tops. Cages were lined with Bed-O-Cob™ (The Andersons Inc., Maumee, OH, USA) or ECOFresh™ bedding (Absorption Corporation, Ferndale, WA). Mice had free access to rodent chow (Purina 5001, 4.5% fat content; Animal Specialties Inc., Hubbard, OR) and water at all times, except during some phases of the CTA studies, as described below. Colony room temperature was 20–22°C, and lights were on a 12:12 h light:dark schedule, with lights on at 0600 h.

Variance estimates from previous experiments investigating behavioral differences between selected lines were used to calculate the number of mice used in each currnt experiment. Power analyses performed on previous data estimated effect size (ω^2) from an analysis of variance (ANOVA) based on Kepple (1991): $\omega^2 = \frac{SS_{treatment} - df_{treatment} \times MS_{error}}{SS_{total} + MS_{error}}$. Sample sizes were determined as a function of desired power (0.80), ω^2, and significance level (α=0.05). A typical effect size for these experiments is ω^2=0.3, with a df=α(n-1)=36. Therefore, a sample size of 12 per line, treatment group and sex provides power

of 0.80 with α=0.05 to detect main effects and interaction effects in behavioral studies such as these.

MA drinking selected mouse lines

The MADR mice were derived from an F2 cross of the C57BL/6J (B6) and DBA/2J (D2) inbred strain mice. The originating B6 and D2 mice were obtained from The Jackson Laboratory (Sacramento, CA) and the F2 cross was created within the VAPORHCS animal facility. A population of 120 F2 mice was tested for voluntary consumption of 20 mg/l and then 40 mg/l MA vs. water, with each concentration offered on 4 consecutive days for 18 h per day, and then mice were selectively bred based on high (MAHDR) and low (MALDR) consumption of the 40 mg/l MA solution. The short-term selective breeding procedures used to create 3 replicate sets of MADR line mice have been fully described (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009). Mice from replicate 2, selection generation 5 (S5) and replicate 3, S5 were used in the current studies.

Taar1 knockout mouse breeding and genotyping.

Taar1 KO mice were obtained from the U.C. Davis Knock Out Mouse Project colony, whose founders were chimeras (KOMP; www.komp.org) and have been previously described (Harkness et al., 2015). Briefly, C57BL/6N embryonic stem cells lacking the entire *Taar1* coding region were injected into BALB/cJ blastocysts, chimeras were backcrossed onto a wildtype B6 background, and offspring were genotyped. Heterozygous (*Taar1* +/-) males and females were bred to produce the +/+ and -/- genotypes used in these experiments.

Drugs and reagents

(+)Methamphetamine hydrochloride (MA) was purchased from Sigma (St Louis, MO, USA). For injection, MA was dissolved in sterile physiological saline (0.9% NaCl; Baxter Healthcare Corporation, Deerfield, IL, USA). Nisoxetine hydrochloride (NISX), fluoxetine hydrochloride (FLUX), and corticosterone (CORT; ≥ 92%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All injections were given intraperitoneally (i.p.) at a volume of 10 ml/kg.

General Procedures

MA-induced plasma ACTH and CORT levels

Two studies were conducted. In the first, ACTH and CORT were measured in plasma from male replicate 2 MADR mice following exposure to saline or MA (1, 2, or 4 mg/kg). In the second study, plasma CORT was measured in male and female MADR replicate 2 and 3, and *Taar1* +/+ and -/- mice following exposure to saline or MA (2 mg/kg). Mice were weighed, isolated from cage mates, and allowed to acclimate for 1 h before injection. In an effort to reduce stress caused by procedural factors not related to MA response, mice were habituated on days 1 and 2 by injection of saline and subsequent isolation in the chambers for 30 minutes before being returned to the home cage and colony room. After 1 h of acclimation on the third day, mice were injected with saline or MA, returned to the isolation chambers, and then euthanized by rapid decapitation at 5, 10, 15, 30, 60, or 120 minutes (study 1), or 60 minutes (study 2) following injection.

Trunk bloods were collected in chilled EDTA coated Vacutainers (BD, Becton, Dickinson and Company, Franklin Lakes, New Jersey) and kept on ice until centrifuged at 3210 g for 20 minutes, within 60 minutes of collection. Plasma in supernatant was collected and samples were stored at 4°C for up to one week before analysis, which was conducted using a [^{125}I] radioimmunoassay kit (MP Biomedical, Solon, OH, USA). Radioimmunoassay was performed following the manufacturer's protocol, adjusted for use of half volumes for all reagents and samples, to accommodate plasma volume. Gamma counts were detected by a 1470 Wizard Automatic Gamma Counter (Wallac, Turku, Finland; Perkin Elmer, Waltham, MA, USA), measuring [^{125}I] degradation during a 5-min period. Counts per minute were averaged from three samples per animal and converted to pg/ml and ug/dl for ACTH and CORT, respectively, using logarithmic functions derived from measurement and conversion of a standard curve. Circulating ACTH and CORT concentrations were then converted to change scores from the saline condition at each time point to obtain a measure of MA effect. Data points that were greater than 2 standard deviations from the group mean were removed from the dataset prior to analysis. There were 21 such data points, and they were approximately equally distributed across groups.

CORT + MA drinking

Voluntary two-bottle choice consumption was measured, using previously described procedures (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009), with the exception that MA- and, CORT- and MA+CORT-containing tubes remained in a constant location, with location on the left vs. right side of the cage

top randomized among mice. This was done to make it easier for the mice to ascertain the location of the drug-containing tube. Food was placed around both tubes. Replicate 2 MAHDR mice were tested in 2 passes of 9-16 mice per treatment group, which were isolate housed and trained during a 48-h period to drink from two water-filled 25-ml graduated glass drinking tubes, fitted with stoppers and straight drinking spouts. Then, on days 1-14, for different groups of mice, the two 25-ml drinking tubes both contained tap water, or one contained tap water and the other contained the “experimental” solution (MA, CORT, or MA+CORT). Mice had 24-h access to a water-containing tube throughout the study, but only 18-h access to the experimental tube. The experimental tube was removed three hours after lights on and replaced three hours before lights off, so that mice had access during the entire dark period and half of the light period.

Mice in the second pass of the study were retained to test the effect of switching CORT and MA+CORT treatment. Following 4 days of water only access, MA and MA+CORT solutions were reversed between groups. Mice in the original MA group were given one tube containing the MA+CORT solution and one water tube, and mice in the MA+CORT group were given one tube containing the MA solution and one water tube. Mice in the CORT group continued to have access to one tube containing CORT and one containing water. Consumption was measured for 10 days (days 19-28) following the same procedure as on days 1-14.

CORT was dissolved following methods described by Gourley and Taylor (2009) and others (Monsey *et al*, 2014). Deionized water was brought to pH 12-

13 using 10 N NaOH. For MA and water groups, solution was neutralized to pH 7.0-7.4 with 10 N HCL before MA was added. For CORT groups, CORT was added at 100 mg/l, and stirred at room temperature for 18-24 hours, and then the solution was neutralized, exactly as for the MA and water solutions. MA was subsequently added to this solution for the MA+CORT group. MA was offered at a concentration of 20 mg/l for 4 days, and then 40 mg/l for 10 days. For the second pass, MA was offered at 40 mg/kg on days 19-28. These concentrations are consistent with those used to collect data for selective breeding (Shabani et al, 2011; Wheeler et al, 2009). The CORT concentration was 100 mg/l and was included with both concentrations of MA in the MA+CORT group. Consumption of this concentration of CORT in solution produces elevated plasma CORT levels of around 30 µg/dl (Karatsoreos *et al*, 2010), which is similar to CORT levels found in MALDR mice (25 µg/dl) following an injection of MA in the current study. Mice were weighed every other day and weights were used with volume (accuracy = 0.2 ml) to calculate MA consumption in mg/kg and volume consumed in ml/kg. Preference ratio was calculated as 18 h MA solution consumed (ml) : 18 h total fluid consumed (ml).

Plasma samples were obtained for CORT measurement, as described above, at the completion of the drinking study, which was day 14 for Pass 1 and day 28 for Pass 2. Pass subgroups were compared at the end of the study to determine whether taking samples on day 14 vs 28 impacted the relationship between MA/CORT intake and CORT levels in plasma. Mice were given access to water and their group-specific experimental tubes, starting at 4:00 pm.

Consumption was recorded and mice were euthanized by rapid decapitation after 8 h of access (12:00 am), a point when mean total consumption was 1.0 ml ± 0.3 for the MA group, 1.3 ml ± 0.2 for the MA+CORT group, and 1.4 ml ± 0.2 for the CORT group. CORT-containing solutions throughout the experiment were tested for CORT content to ensure CORT stability in solution.

NISX- or FLUX-induced CTA

The purpose of this study was to determine whether MAHDR and MALDR mice are differentially sensitive to the aversive effects of the NET and SERT blockers. Procedures were consistent with those used to measure MA-induced CTA (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009). Replicate 2 MADR mice were isolate-housed and acclimated to water restriction (2 h of water per day) on days 1-4. They were then introduced to the novel taste of a 0.2M NaCl solution on day 5, during a 1-h access period. Conditioning trials then occurred every other day, during which mice were given access for 1 h to the NaCl solution (days 7, 9, 11, 13, and 15), and were then immediately injected with saline, NISX (25 or 50 mg/kg) or FLUX (12.5, 25, or 50 mg/kg). These doses of NISX and FLUX were based on other reports of doses used to induce CTA in mice (Jones *et al*, 2009; 2010). Water was available for 30 minutes, 3 h following injections, to avoid dehydration. On intervening non-conditioning days, water was available for 2 h and no injection was administered.

Effect of prior treatment with NISX or FLUX on MA-induced CTA

The purpose of this study was to determine the potential effect of repeated monoamine blockade on subsequent MA-induced CTA. Replicate 2 MADR mice

were tested using methods similar to those used by Jones et al. (2009; 2010). Mice were first introduced to drinking from graduated drinking tubes (days 1-2), then saline, NISX (50 mg/kg) or FLUX (50 mg/kg) was administered, once every other day for a total of 5 saline or drug treatments (10 total days). The MA-induced CTA conditioning procedure was then performed (days 13-23). The 50 mg/kg dose was chosen because both blockers produced significant CTA when administered at this dose.

Data analysis

Data were analyzed by ANOVA. Possible between-groups factors were selected line, sex, dose and time. Day was a within-subject factor in the CTA and drinking studies. Significant three-way interactions were further examined by two-way ANOVAs of relevant factors. Sources of significant two-way interactions were examined using simple main effect analysis, and the Neuman–Keuls test was used for post hoc mean comparisons. Alpha level was set at < 0.05, and statistical analyses were performed using the Statistica 12 software package (StatSoft, Inc., Tulsa, OK). Statistical results are presented in the figure legends.

Results

Stress axis response to MA

Acute injection of MA, regardless of dose, increased plasma CORT in male MALDR mice, but not male MAHDR mice of replicate 2 (Fig. 3.1). In addition, male MALDR mice had a more sustained ACTH response than male MAHDR mice. Plasma ACTH levels peaked at 10-15 minutes, whereas plasma CORT levels peaked at 60 minutes following MA injection. In a second study that

examined CORT response in both male and female replicate 2 and 3 mice, *Taar1* +/+ and MALDR mice, which both have functional TAAR1, showed increases in plasma CORT, following a 2 mg/kg MA treatment. Their CORT levels were significantly different from those of *Taar1* -/- and MAHDR mice, which did not show elevations in CORT level and do not have functional TAAR1 (Fig. 3.2). In replicate 3 mice, female MALDR mice exhibited a significantly greater CORT response, compared to male MALDR mice. However, there were no significant effects involving sex in replicate 2 or in the transgenic mice. Additionally, there were no significant changes in plasma CORT levels in MA treated replicate 2 or 3 MAHDR mice or *Taar1* -/- mice, following 2 mg/kg MA treatment, compared to saline-treated mice of the same genotype.

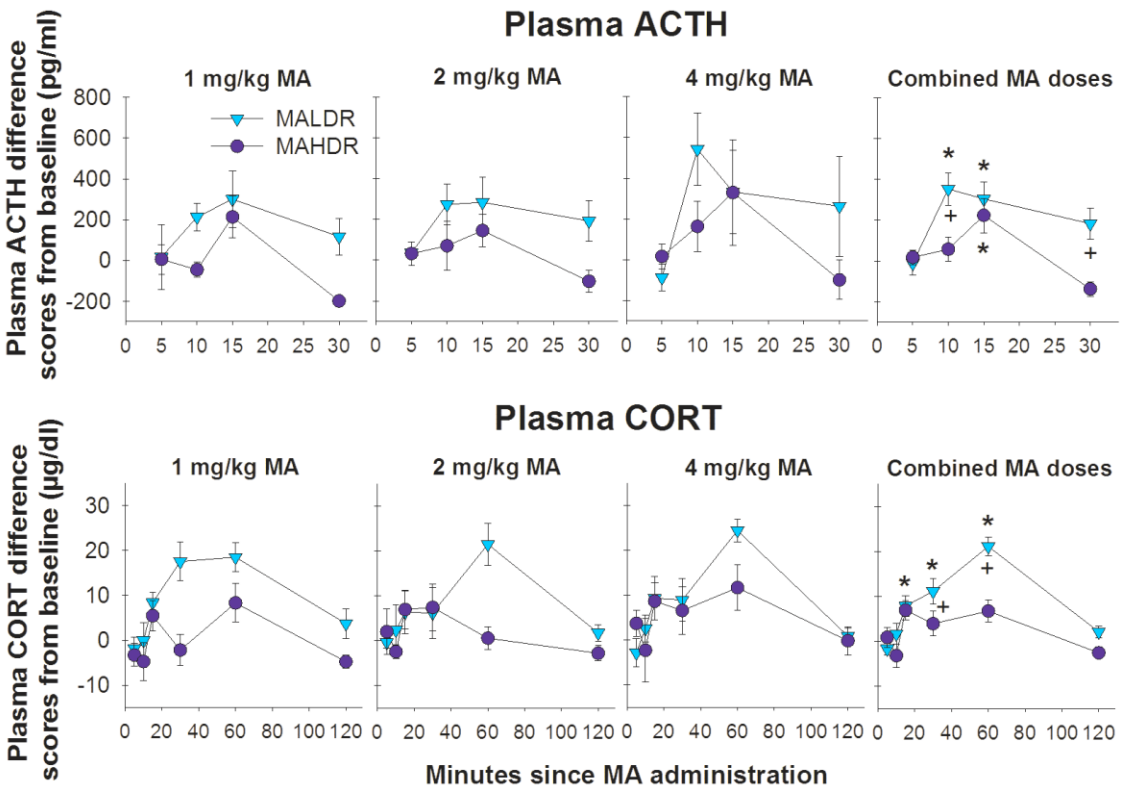

Figure 3.1. Plasma adrenocorticotropic hormone (ACTH) and corticosterone (CORT) difference values from saline baseline after administration of several doses of MA. Data are shown for each methamphetamine (MA) dose, but there were no dose-dependent effects. The same data, combined across MA doses (1, 2, and 4 mg/kg), are shown in far right panels. MAHDR mice were less sensitive to the stress axis-activating effects of MA than MALDR mice. Saline group means at each time point were subtracted from individual MA values to illustrate the effect of MA. **Top panels**: Blood was collected at 5, 10, 15, or 30 min post-injection and analyzed for ACTH. A significant line x time interaction [$F(3,138)=3.01$ $p<0.05$] was found. Simple main effect analysis of the line x time interaction identified a significant effect of time for both MAHDR [$F(3,138)=4.26$

p<0.01] and MALDR [F(3,138)=5.72 *p*<0.005] mice. In MAHDR mice, plasma ACTH levels above baseline were higher at 15 minutes post injection, compared to 5 minutes ($p < 0.05$). In MALDR mice, plasma ACTH levels were higher at 10 and 15 minutes after injection, compared to 5 minutes (p's<0.05). MALDR mice had significantly higher baseline-corrected ACTH levels at 10 and 30 minutes post injection, compared to MAHDR mice at the same time points (p<0.05). **Bottom panels**: Blood was collected for CORT samples at 5, 10, 15, 30, 60, or 120 min post-injection. A significant line x time interaction [F(5,199)=3.87 *p*<0.005] was found. Simple main effect analysis of the line x time interaction identified a significant effect of time for both MAHDR [F(5,289)=2.69 *p*<0.05] and MALDR [F(5,289)=13.46 *p*<0.0001] mice. However, no significant mean differences were found for MAHDR mice. In MALDR mice, plasma CORT levels above baseline were higher at 15, 30, and 60 minutes after injection, compared to 5 minutes. In addition, baseline-corrected CORT levels in MALDR mice were higher at 30 and 60 minutes, compared to levels in MAHDR mice (all p's<0.05). Shown are means ± SEM. N=12 per line, dose and time point, age 54-165, replicate 2.

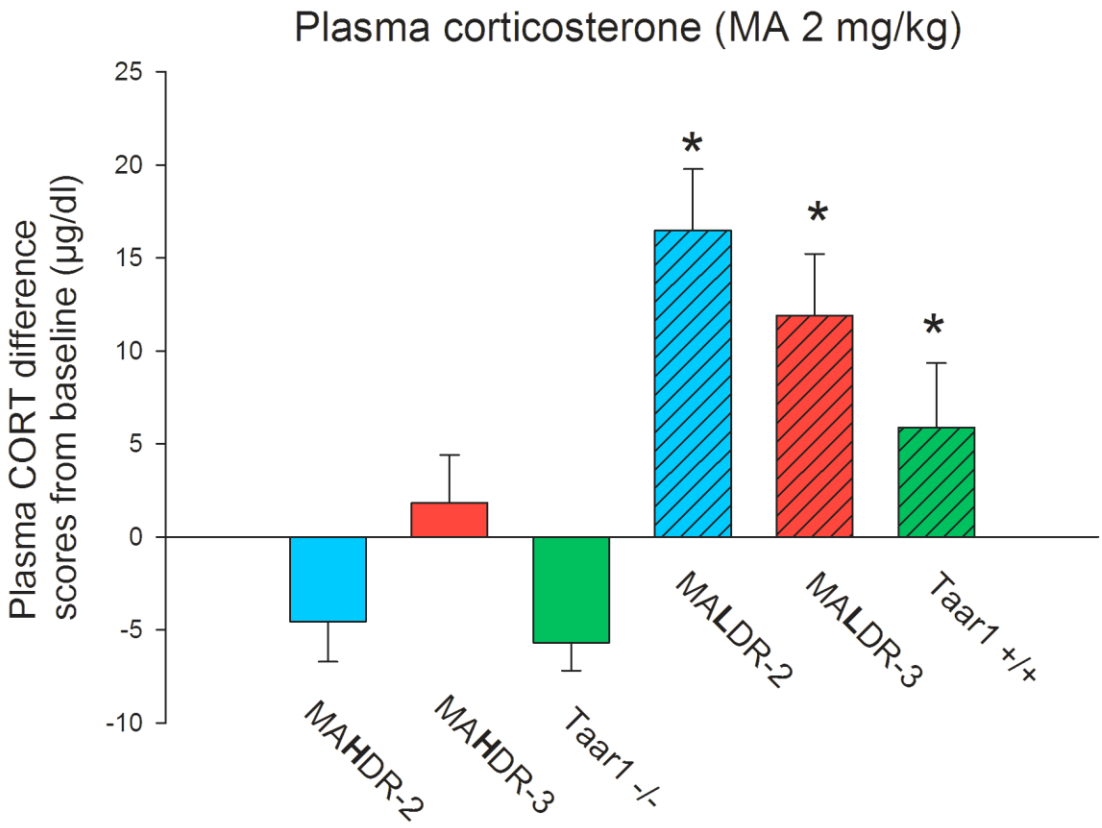

Figure 3.2. Plasma corticosterone (CORT) difference values from saline baseline 60 minutes after administration of 2 mg/kg methamphetamine (MA) in MADR and *Taar1* transgenic mice. MAHDR and *Taar1* -/- mice were insensitive to the HPA axis-activating effects of that dose of MA, compared to MALDR and *Taar1* +/+ mice. Saline group means were subtracted from individual MA values. A significant main effect of genotype was found within the MADR-2 lines (blue bars) [$F(1,14)=28.39$ $p<0.005$], MADR-3 lines (red bars) [$F(1,59)=5.24$ $p<0.05$], and the TAAR1 lines (green bars) [$F(1,17)=10.15$ $p=0.005$]. A significant sex x line interaction was found for the replicate 3 MADR mice [$F(1,61)=5.6$ $p<0.05$]; female MALDR mice had significantly higher baseline-corrected CORT levels than their male counterparts, but both sexes of MALDR mice showed an

elevation, compared to saline treated mice (data not shown). Note: *Taar1* mice were in short supply and before testing for MA-induced CORT levels, had been included in another study in which MA was offered in a two-bottle choice drinking procedure for 8 days. The mice were allowed a 2-week rest interval between the studies. Shown are means ± SEM. MADR-2: N=8 per line, age 67-110 days; MADR-3: N=6-10 per line, age 87-119 days; *Taar1* transgenic: N=9-16 per line, age 130-403 days. *: $p<0.05$ for the difference between MALDR compared to MAHDR mice (per replicate) and *Taar1* +/+ compared to *Taar1* -/- mice.

Effect of CORT on MA drinking

Fig. 3.3a & b show MA consumption in male and female replicate 2 MAHDR mice from passes 1 and 2. When CORT was included in the MA solution, consumption of MA for the 40 mg/l concentration significantly decreased over days in male, but not female MAHDR mice. There was no impact of CORT on MA consumption for the 20 mg/l MA solution. There was a significant interaction of sex x CORT treatment x day for 18-h water consumption (ml/kg) in MA vs MA+CORT drinking mice [$F(13,351)=1.8$, $p<0.05$]. Follow-up analyses identified a significant effect of day for water consumption only in female mice of the MA+CORT group [$F(13,52)=2.03$. $p<0.05$]; water consumption increased across days in these mice.

In the second pass, inclusion of CORT in the MA solution on days 1-14 significantly decreased consumption of MA over days and there was not significant effect of sex (Fig. 3.3c). When treatment groups were switched to offer MA mice the MA+CORT solution and vice versa on days 19-28, consumption no longer differed between the groups. When data from the 2 passes of the experiment were combined, the effect of CORT on intake was seen only in male mice; however, when preference was examined, there was a significant difference between the MA and MA+CORT groups that was not sex-specific. Therefore, data are shown for the 2 sexes combined in Fig. 3.4, and indicate significantly lower preference in the MA+CORT group. Preference scores for the CORT alone solution offered vs. water were similar to those for mice offered

water in both tubes and were about 0.5, indicating that there was no preference or aversion for the CORT-containing solution.

There was no significant impact of whether sampling occurred on day 14 vs day 28. Plasma CORT levels were significantly elevated in mice consuming CORT or MA+CORT solutions, compared to water (Fig. 3.5). Plasma CORT levels in the MA+CORT group were also significantly higher than in the MA group. CORT levels did not significantly differ by sex.

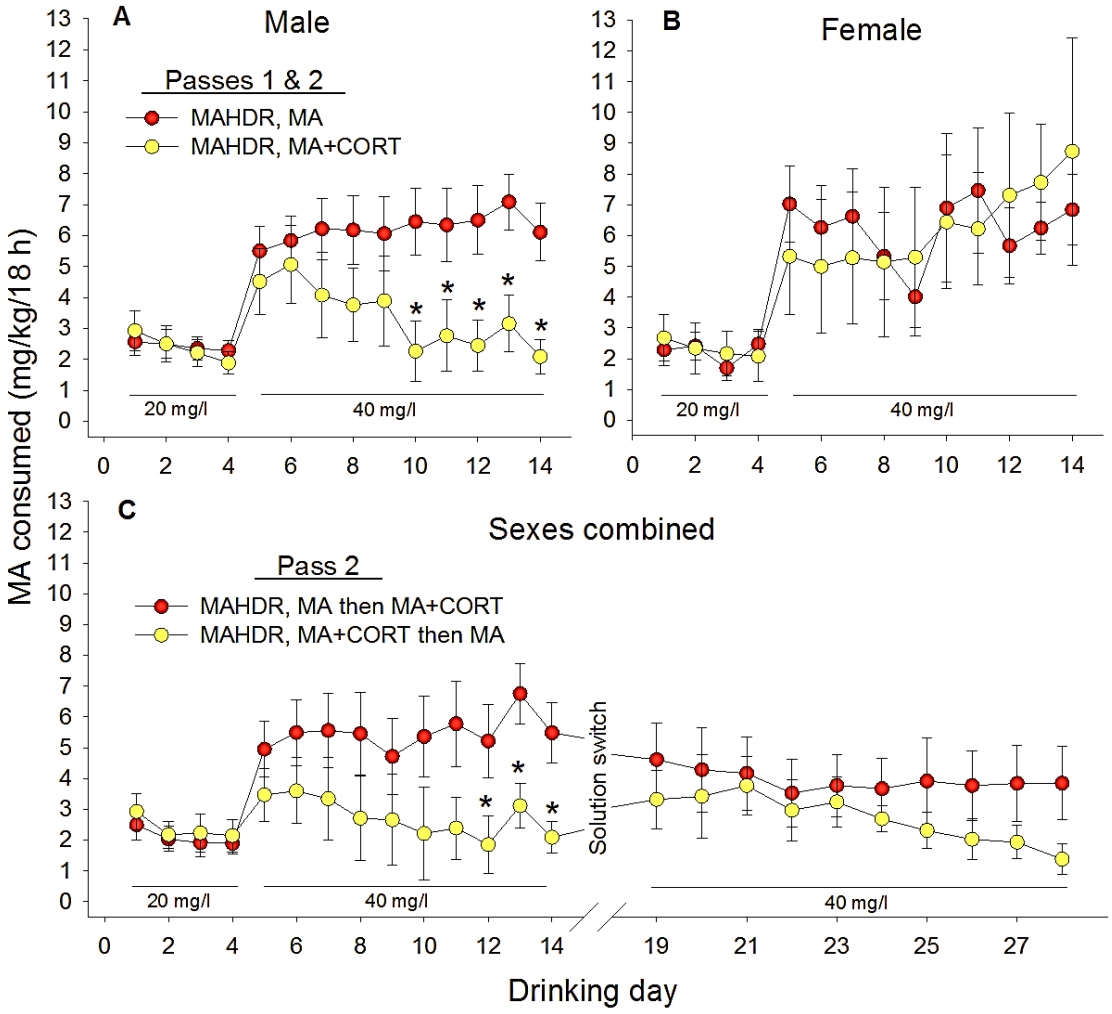

Figure 3.3. Methamphetamine (MA) consumption decreased over days in male, but not female, MAHDR mice offered MA in solution with corticosterone (CORT). (**a & b**) MA consumption (mg/kg) by MAHDR mice from Pass 1 & 2 combined, increased with increasing MA concentration from 20 mg/l (days 1-4) to 40 mg/l (days 5-14). A significant day x sex x CORT treatment interaction was found [$F(13,351)=2.0$ $p<0.05$], and follow-up analyses indicated a significant day x CORT interaction in (**a**) male [$F(13,234)=4.3$ $p<0.000005$], but not (**b**) female, mice. (**c**) A subset of mice from the MA and MA+CORT groups (Pass 2)

continued MA consumption for another 10 days (days 19-28) after their group assignments were switched. A significant day x CORT treatment interaction was found in MA drinking MAHDR mice during the first 14 days [$F(13,195)=3.6$ $p<0.0001$], but not after solutions were switched on days 19-28. Shown are means ± SEM. Age 52-108 days, replicate 2. (**a & b**) N=5-11 per sex and group. (**c**) N=3-6 per sex and group (N=9 per group, when collapsed on sex as shown here). *: $p<0.05$ for the difference in MA consumed by the MA group, compared to MA+CORT group on specific day.

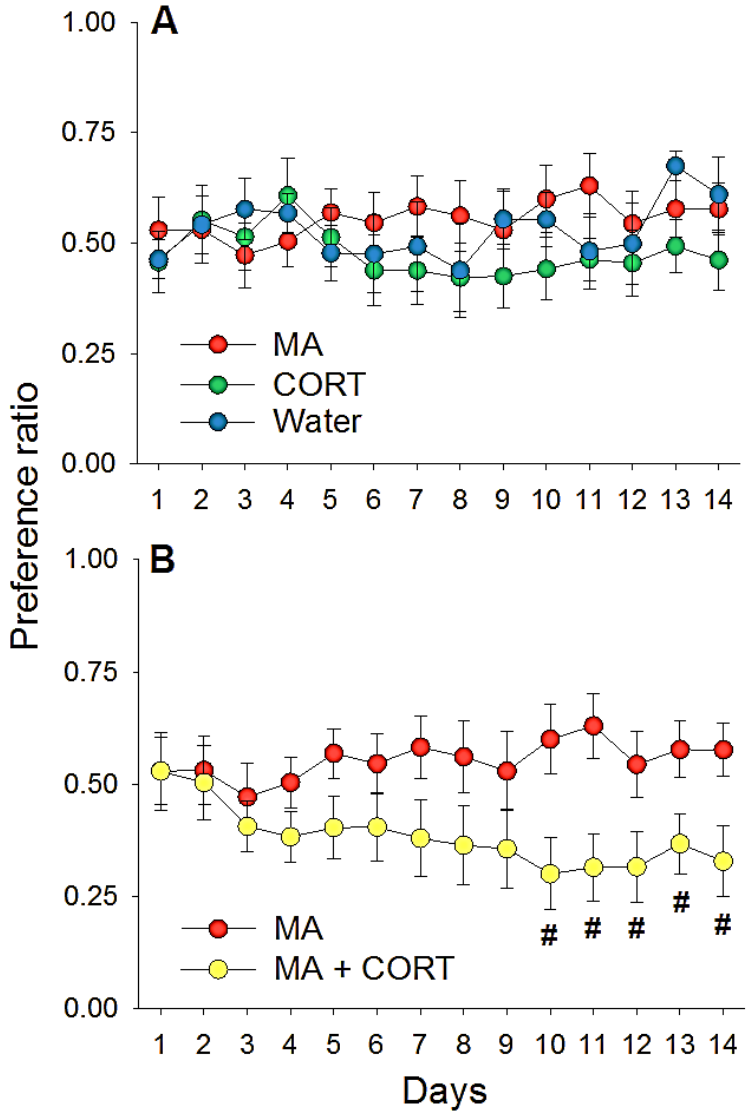

Figure 3.4. Methamphetamine (MA) preference over water decreased over days in MAHDR mice offered MA in solution with corticosterone (CORT). (**a**) Water, CORT, and MA solution preferences were not significantly different in MAHDR mice. (**b**) Preference values for MA and MA+CORT groups show that preference values declined over days when CORT was present in the MA solution. A

significant day x treatment effect was found in mice drinking MA or MA+CORT [$F(13,377)=2.02$; $p<0.02$]. There was no significant effect of sex, so data are shown for males and females combined. The same MA data are shown in both panels for comparison purpose. Shown is mean ± SEM. N=3-11 per sex and group (N=8-16 per group, when collapsed on sex as shown here), age 52-108 days, replicate 2. #: $p<0.05$ for the difference in preference from MA group on specific day.

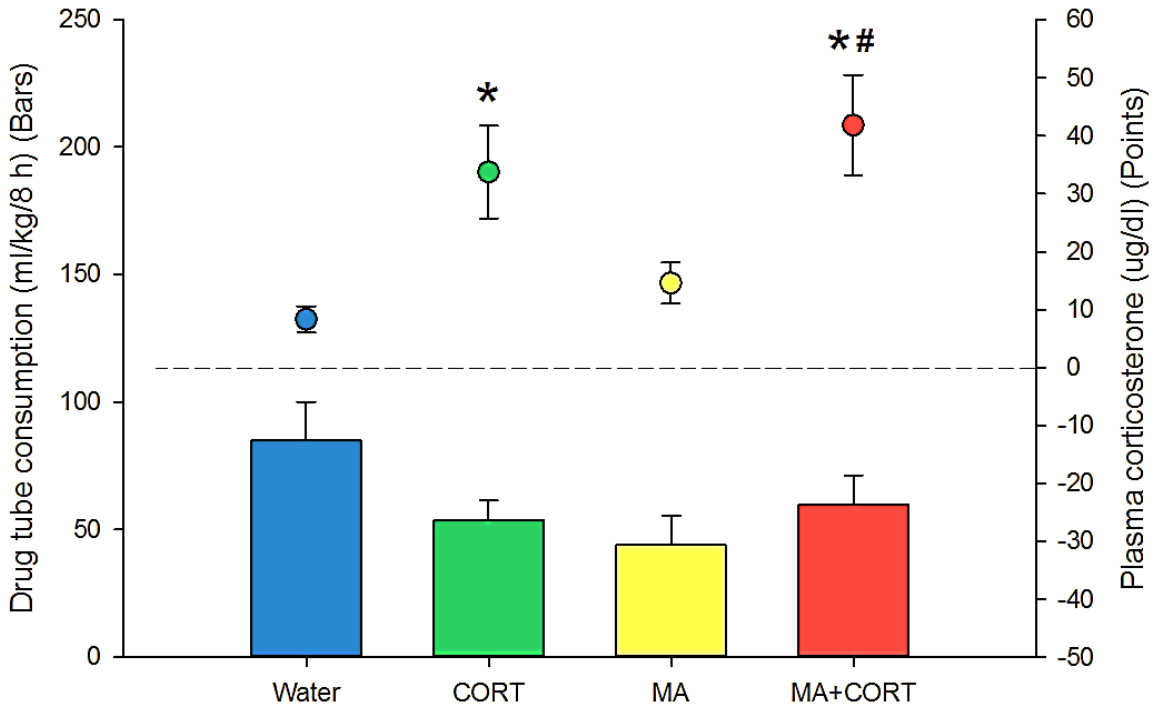

Figure 3.5. Plasma corticosterone (CORT) levels 8 h after consuming water, CORT, methamphetamine (MA) or MA+CORT in replicate 2 MAHDR mice. A main effect of treatment was found for plasma CORT levels in MAHDR mice consuming solutions with or without added CORT [$F(3,52)=4.47$; $p<0.01$]. Mice offered CORT in solution with water had significantly elevated plasma CORT levels, shown as points (right axis), compared to mice offered water alone, and CORT levels in mice offered MA+CORT were significantly higher than in mice offered either water or MA. There was no significant effect of solution on total 8 h consumption (ml/kg/8 h); shown as bars (left axis). CORT content was stable in CORT and MA+CORT solutions, measured throughout the drinking experiment (data not shown). Shown are means (±SEM). N=3-11 per sex and group (N=8-16

per group, when collapsed on sex as shown here), age 52-108 days, replicate 2. *: $p<0.05$ for the difference between plasma CORT level compared to the MA group. #: $p<0.05$ for the difference between plasma CORT level compared to the water group.

NISX-induced CTA

NISX treatment dose-dependently reduced NaCl consumption across days (Fig. 3.6). This effect was found only for the highest dose of NISX. MALDR mice treated with 50 mg/kg NISX consumed significantly less NaCl than MAHDR mice on the first two days after NISX pairing (Test day 9 and 11 in Fig. 3.6); however, MALDR mice also consumed significantly less NaCl solution prior to NISX treatment (Test day 7 in Fig. 3.6), therefore, the difference in NaCl consumption cannot be interpreted as a difference in response to NISX treatment. By the fourth and fifth days of NaCl access, consumption did not differ between the lines and eventually reached near-floor levels. Treatment with 25 mg/kg NISX also decreased mean intake of NaCl over days; however, this effect was identical in both MAHDR and MALDR mice. No significant effects of sex were found at any dose of NISX.

FLUX-induced CTA

FLUX treatment also dose-dependently reduced NaCl consumption across days (Fig. 3.7). In this study, groups and lines were well matched for NaCl consumption prior to FLUX treatment. Groups treated with the 12.5 mg/kg dose of FLUX decreased their consumption of the NaCl solution, but MALDR mice exhibited significantly reduced consumption earlier than MAHDR mice, and consumed significantly less NaCl than MAHDR mice on every day following the FLUX pairing. Similar patterns of response were found for the sexes, and data are shown collapsed on sex in Fig. 3.7; however, female MAHDR mice treated with 12.5 mg/kg FLUX developed greater CTA than male MAHDR mice

($p<0.05$), and female MALDR mice treated with 50 mg/kg FLUX developed greater CTA than male MALDR mice ($p<0.005$). Consumption of NaCl decreased to nearly 0 ml in both MADR lines treated with 25 or 50 mg/kg FLUX, and the effect of FLUX did not differ between the lines.

Effect of Prior Treatment with NISX on MA-induce CTA

Consistent with published results (Shabani *et al*, 2012b; Wheeler *et al*, 2009), MA treatment produced a CTA to NaCl in MALDR, but not MAHDR, mice (Fig. 3.8.) Prior treatment with NISX had no effect on the development of MA-induced CTA. There were no significant effects of sex.

Effect of Prior Treatment with FLUX on MA-induced CTA

Again, MA treatment produced a CTA to NaCl in MALDR, but not MAHDR, mice. Prior treatment with FLUX reduced the magnitude of MA-induced CTA in MALDR mice on the days after the first two MA pairings (Fig. 3.9). There were no significant effects of sex.

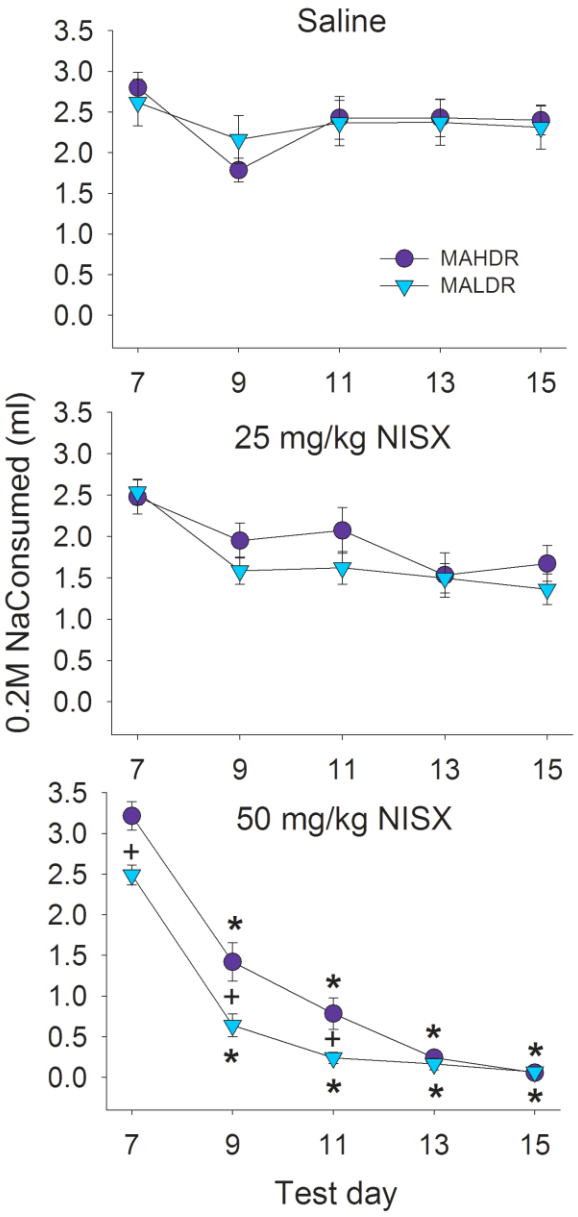

Figure 3.6. Nisoxetine (NISX)-induced CTA was similar in MAHDR and MALDR mice. There was a significant dose x line x day interaction [$F(8,424)=1.94$, $p=0.05$]. Follow up analyses identified a significant effect of day in saline [$F(4,140)=3.96$, $p<0.005$], and 25 mg/kg NISX [$F(4,144)=12.14$, $p<0.0001$] groups. There was a significant line x day interaction for 50 mg/kg NISX

[F(4,148)=5.49, p<0.0005]. MALDR mice consumed less NaCl than MAHDR mice on Test days 7, 9 and 11. Shown are means ± SEM. N=9-10 per sex, line and dose (N=18-20 per line and dose, when collapsed on sex as shown here), age 81-114 days, replicate 2. *: p<0.05 for the difference in NaCl consumption on day indicated from day 7. +: p<0.05 for the difference in NaCl consumption between MAHDR and MALDR mice.

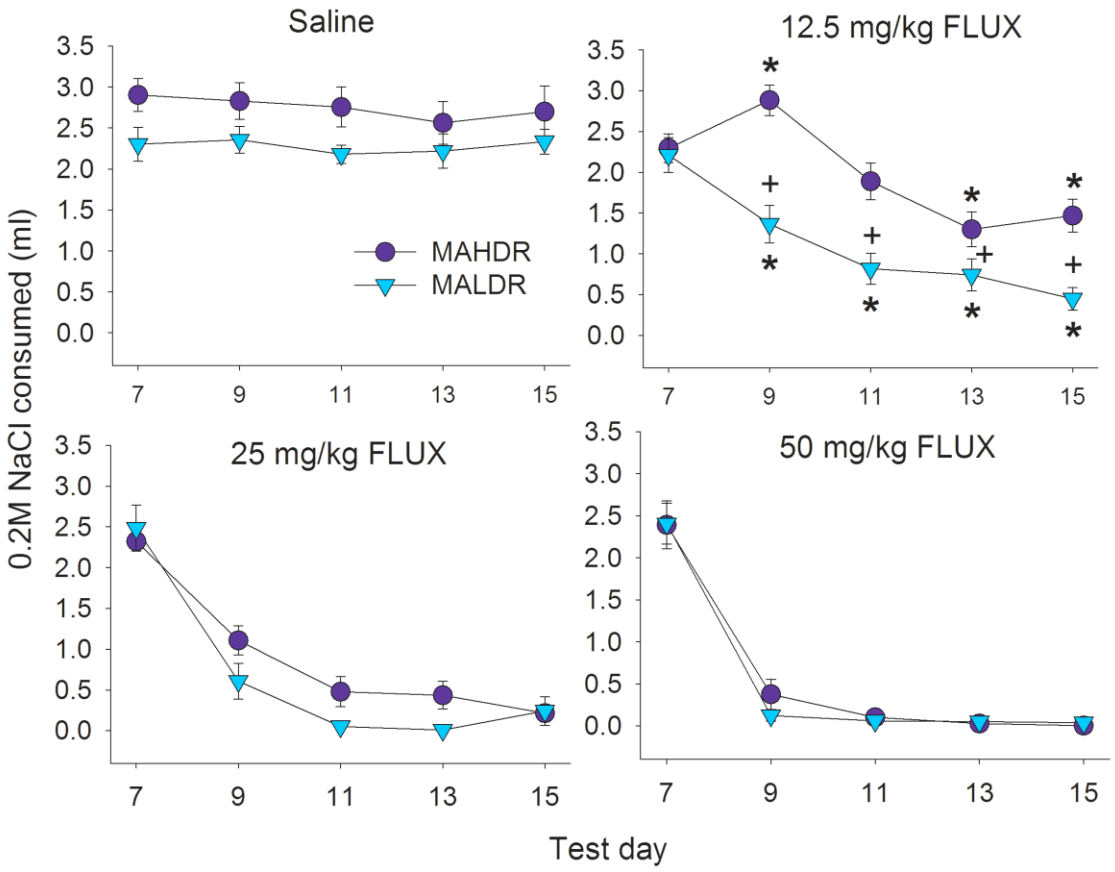

Figure 3.7. Sensitivity to fluoxetine (FLUX)-induced CTA in the MADR lines. MALDR mice were more sensitive to the aversive effects of FLUX at 12.5 mg/kg. There was a significant dose x line x day interaction [$F(12,488)=1.94$, $p<0.05$]. There was also a significant line x day interaction at the lowest dose of FLUX (12.5 mg/kg) [$F(4,154)=7.07$, $p<0.00005$], and MALDR line mice consumed less NaCl than MAHDR mice after the first FLUX pairing. Female MAHDR mice developed significantly greater CTA over days compared to male mice treated with 12.5 mg/kg FLUX [$F(4,76)=2.98$, $p<0.05$], and female MALDR mice treated with 50 mg/kg FLUX compared to male MALDR mice [$F(4,52)=4.64$, $P<0.005$].

However, the pattern of response to treatment was similar between sexes and data are therefore collapsed on sex. Shown are means ± SEM. N=6-11 per sex, line and dose (N=13-21 per line and dose, when collapsed on sex as shown here), age 68-93 days, replicate 2. *: $p<0.05$ for the difference between NaCl consumption on day indicated from baseline drinking. +: $p<0.05$ for the difference in NaCl consumption between MAHDR and MALDR lines.

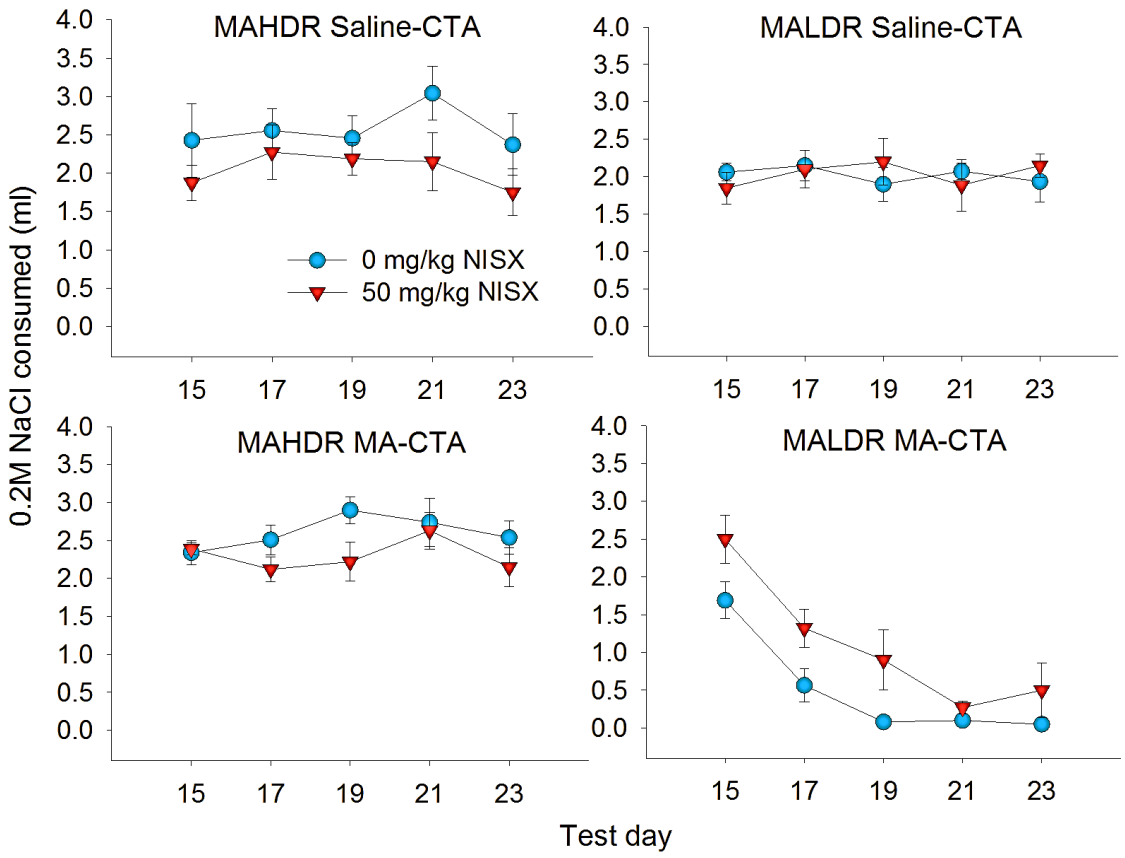

Figure 3.8. Repeated treatment with nisoxetine (NISX) for 10 days prior to methamphetamine (MA)-induced CTA did not significantly impact CTA development. No significant effect of previous NISX treatment was seen in MAHDR (left) or MALDR (right) mice in saline conditioned (top) or 2 mg/kg MA conditioned (bottom) groups. A significant MA treatment x line x day interaction [$F(4,256)=7.46$, $p<0.00005$] was found. MALDR mice were more sensitive than MAHDR mice to MA-induced CTA. Shown are means ± SEM. N=3-5 per sex, line and dose (N=7-10 per line and dose, when collapsed on sex as shown here), age 57-120 days, replicate 2.

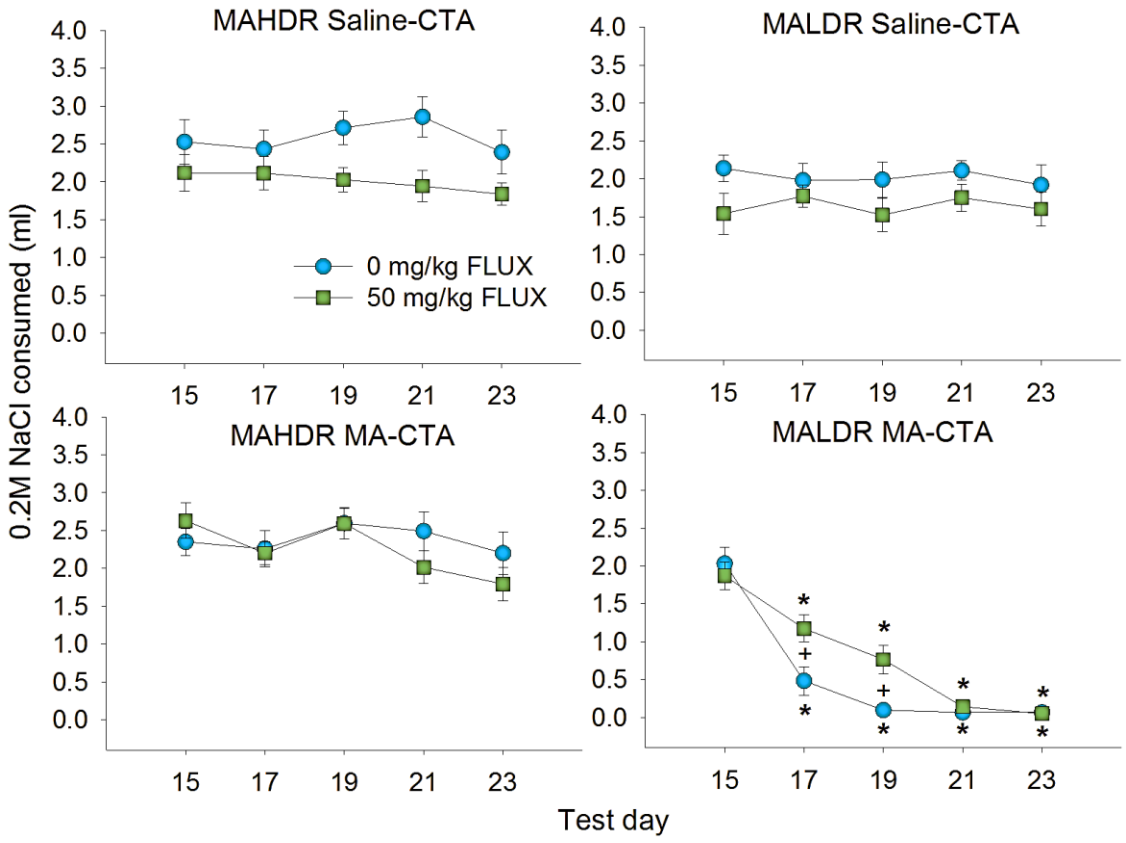

Figure 3.9. Repeated treatment with fluoxetine (FLUX) for 10 days prior to methamphetamine (MA)-induced CTA decreased sensitivity to MA-induced aversion in MALDR mice (right). No significant effect of previous FLUX treatment was seen in MAHDR mice (left) in saline conditioned (top) or 2 mg/kg MA conditioned (bottom) groups. A significant day x FLUX treatment x line interaction was found for the MA conditioned [$F(4,180)=2.44$, $p<0.05$], but not saline conditioned, group. Follow up analyses identified a significant line x day interaction [$F(4,188)=18.33$, $p<0.0001$] in the MA conditioned mice. MALDR mice developed significantly greater MA-induced CTA over days than MAHDR mice, regardless of FLUX treatment. Additionally, a significant FLUX treatment x day interaction [$F(4,88)=4.3$, $p<0.001$] was found in MA conditioned MALDR mice.

When treated with FLUX, the development of MA-induced CTA was slowed in MALDR mice. Shown are means ± SEM. N=4-7 per sex, line and group (N=10-14 per line and group, when collapsed on sex as shown here), age 79-120 days, replicate 2. *: $p<0.05$ for the difference between NaCl consumption on day indicated from baseline drinking within group. +: $p<0.05$ for the difference in NaCl consumption between saline and FLUX treatment groups.

Discussion

MA aversion in the MADR and *Taar1* transgenic lines is correlated with TAAR1 function, which may limit voluntary consumption of MA in mice expressing functional *Taar1* (Harkness *et al*, 2015). Previously, we presented data supporting a role for TAAR1 function in MA intake, MA-induced hypothermia and sensitivity to MA-induced CTA (Harkness *et al*, 2015). Here, we found that MA-induced elevation of plasma CORT corresponded with low MA intake and *Taar1* genotype, such that only mice with a *Taar1* allele expressing functional TAAR1 exhibited elevated CORT. Furthermore, consumption of CORT increased plasma CORT levels to a greater extent in mice also consuming MA, and decreased preference for MA in MAHDR mice. Overall, these data provide evidence that elevated circulating CORT may contribute to an aversive effect of MA that contributes to limiting MA consumption, which is seen specifically in mice expressing functional *Taar1*. Finally, blockade of NET does not appear to result in a differential aversive response in the MADR lines or to alter MA-induced CTA, whereas SERT may play a partial role in sensitivity to MA-induced aversion.

Plasma ACTH and CORT were significantly elevated in MALDR, but not MAHDR, mice following MA treatment (Fig. 3.1). CORT levels were also elevated by MA treatment in *Taar1* +/+, but not -/-, mice (Fig. 3.2). Data for this effect of MA were consistent in two independent sets of the MADR lines, and elevated CORT corresponded with TAAR1 function. These are the first data to suggest that TAAR1 function may affect HPA response to MA, although it was previously reported that intracerebral and systemic administration of two thyronamine

compounds (T_1AM and T_0AM) with agonist actions at TAAR1 increased plasma CORT levels in rats (Klieverik *et al*, 2009). The effect of MA on CORT levels in MALDR mice was somewhat larger than in *Taar1* +/+ mice, suggesting that selection for low MA intake may have altered additional genetic mechanisms relevant to regulation of HPA axis response to MA.

In general, it appears that thermal and stress hormone responses to MA are similarly correlated with *Taar1* genotype. Though there are some differences with regard to the effects of specific doses of MA between the second and third replicates of MADR mice – for example, replicate 2 MALDR mice became more hypothermic to lower doses of MA than replicate 3 MALDR mice (Harkness *et al*, 2015) – the responses are qualitatively similar. Plasma CORT levels in response to 2 mg/kg MA also appear to be higher for replicate 2, compared to replicate 3, MALDR mice (Fig. 3.2), consistent with the difference in magnitude of the hypothermic response in these replicate lines. There were no sex differences in the effect of MA on plasma CORT levels in replicate 2 MADR mice, or *Taar1* transgenic mice, but female replicate 3 MALDR mice exhibited a greater plasma CORT response to MA than did male replicate 3 MALDR mice, although the increase in plasma CORT levels occurred in both sexes. These magnitude differences may reflect the effect of genetic differences between the replicate 2 and 3 selected lines. Because the breeding populations are relatively small, it is not unexpected that one or more polymorphic alleles relevant to the selection phenotype could be lost in one replicate of selection of mice, while being retained in the other.

The addition of CORT to the MA drinking solution decreased the preference for the MA-containing solution in replicate 2 MAHDR mice, and this effect was not dependent on sex. However, CORT decreased MA consumption (mg/kg) in male mice only. Water consumption in female MA+CORT consuming mice tended to increase over time, while male MA+CORT water consumption was stable. Thus, it is possible that increased water consumption by female mice resulted in no sex difference in MA+CORT preference, which was apparent when considering MA+CORT consumption. Alternatively, it is possible that male MAHDR mice are more sensitive than female mice to the presence of CORT in the MA+CORT solution. For example, decreased MA consumption may have been found in female mice if a higher concentration of CORT had been added to the MA solution.

In a subset of mice, when CORT was no longer present in the MA solution, consumption of that group increased to levels near those of mice previously consuming MA alone (Fig. 3.3c). However, consumption did not decrease in mice that had been consuming MA when they were offered a MA+CORT solution on days 19-28. This suggests that increased CORT may be important to learning aversive qualities during initial exposures to MA, but that the impact may decrease after MA experience without CORT elevation has been obtained.

Consumption of solutions containing CORT significantly elevated plasma CORT levels in a non-sex-dependent manner (Fig. 3.5). However, plasma samples were collected at only a single time point, which was 8 h into the

drinking period, a time point when mice from all groups had consumed enough of the solutions for us to feel confident that we could potentially see a difference in CORT levels. It is possible that samples taken at other time points would have been more informative regarding plasma CORT levels. This was not done in the current study to avoid disturbance of the drinking behavior, but is an important question for future work.

The actions of MA on NE and 5-HT systems (Bunzow *et al*, 2001; Fleckenstein *et al*, 2007; Wolinsky *et al*, 2007), implications for NET and SERT in aversive response to cocaine, (Jones *et al*, 2009; Rothman *et al*, 2001; Serafine and Riley, 2009), and expression differences of the genes encoding NET and SERT between the MADR lines (Wheeler *et al*, 2009), made NET and SERT function interesting targets for the study of MA aversion in MADR mice. Blockade of NET, and of SERT were each sufficient to induce CTA in both MADR lines. CTA developed faster in MALDR compared to MAHDR mice at the lowest dose of the SERT blocker tested (Fig. 3.7), but both lines showed significant CTA. A difference in NaCl consumption was also seen between MALDR and MAHDR mice in the highest dose group for the NET blocker (Fig. 3.6), but there was an initial line difference in NaCl consumption between the lines for these particular groups that was not seen for other studies here or previously (Shabani *et al*, 2012b; Wheeler *et al*, 2009). MAHDR mice were sensitive to transporter blocker-induced CTA. However, they are not sensitive to MA-induced CTA, as shown here and in previous studies (Shabani *et al*, 2012b; Wheeler *et al*, 2009). This indicates that genetic susceptibility to the conditioned aversive effects of the

transporter blockers involve different mechanisms, compared to those underlying the conditioned aversive effect of MA.

However, that SERT blockade altered the development of MA-induced CTA suggests some involvement of SERT in this phenotype. Reductions in magnitude or rate of acquisition of CTA produced by a drug, following pre-exposure to another drug, have been speculated to reflect overlapping mechanisms of action (Fox *et al*, 2006; Jones *et al*, 2009; Kunin *et al*, 2001). If the pre-exposure drug interacts with a target shared with the CTA-inducing drug, then the pre-exposure drug may induce adaptations that alter the magnitude of the subsequent drug effect. Data from studies in which the pre-exposure and CTA-inducing drug were the same support this idea. For example, pre-exposure to cocaine prior to induction of CTA with cocaine reduced the rate of acquisition or magnitude of CTA (Davis and Riley, 2007; Riley and Diamond, 1998). Furthermore, pre-exposure to either NISX or FLUX, but not the DAT inhibitor, GBR-12909, before CTA induction by cocaine attenuated the magnitude of cocaine-induced CTA (Jones *et al*, 2009). This suggests a role for NET and SERT in cocaine-induced CTA.

To determine if a similar outcome would be seen for MA-induced CTA, we tested the effects of NISX and FLUX pre-exposure in the MADR lines. When mice were repeatedly treated with the NET blocker prior to MA-induced CTA, there was no significant effect on development of MA-induced CTA (Fig. 3.8). However, when the SERT blocker was repeatedly administered prior to MA-induced CTA, the rate of MA-induced CTA acquisition was slowed in MALDR

mice (Fig. 3.9). This may indicate that SERT plays a role in MA-induced CTA in MALDR mice. However, the effect of the SERT blocker on MA-induced CTA was considerably smaller than the effect of non-functional TAAR1, which resulted in the complete absence of MA-induced CTA (Harkness *et al*, 2015). The current data also suggest a difference in mechanisms involved in MA- vs. cocaine-induced CTA, since Jones et al (2009) found that blockers of both SERT and NET altered the development of cocaine-induced CTA. It is possible though, that the different genotype used by Jones et al (2009), which was ND4 Swiss–Webster albino mice (Harlan Sprague–Dawley), could have affected their results. Additionally, we did not investigate effects of repeated NET or SERT blockade, or repeated MA on transporter expression or function, which may be associated with MA-induced CTA. Future investigations should measure transporter density and MA binding in mice following the same CTA procedures used here. In the current studies, we used both the MAHDR and MALDR lines because of the possibility that the blockers could enhance sensitivity to the aversive effects of MA. However, no effects of NISX or FLUX pretreatment were seen in the MAHDR line, which is insensitive to MA-induced CTA. Based on the findings of Jones et al (2009), and because we have not seen MADR line differences in expression of the DAT encoding gene, *Slc6a3,* a DAT inhibitor was not included in the current investigation because it was not expected that DAT blockade would reduce MA-induced CTA.

Differing TAAR1 functionality in the MAHDR and MALDR lines could explain line differences in stress axis activation following MA exposure. TAAR1

modulates monoamine transporters in mice and primates by phosphorylation and internalization of transporters (Miller, 2011, 2012; Revel *et al*, 2011; Xie and Miller, 2008, 2009). *Taar1*-/- mice exhibit lower basal levels, and greater amphetamine-induced release of DA, 5-HT, and NE in the striatum compared to WT mice (Lindemann *et al*, 2008; Pringle *et al*, 2008; Wolinsky *et al*, 2007). MAHDR mice, which are natural *Taar1* mutants, (Harkness *et al*, 2015), also have lower basal dopamine levels in the NAcc and mPFC, and higher DA release following MA, but only in the mPFC, not NAcc (Lominac *et al*, 2014). It is possible that MAHDR mice display an altered dopamine phenotype because of a lack of TAAR1 activity. Centrally, NE activity influences the central corticotropin-releasing factor (CRF) system through projections to the locus coeruleus (LC), amygdala, paraventricular nucleus of the hypothalamus (PVN), and forebrain (Makino *et al*, 2002). Peripherally, NE is produced by the adrenal medulla through sympathetic stimulation (Sedvall *et al*, 1968) and local effects of CORT (Iuvone *et al*, 1977). NE positively feeds back to the pituitary and increases the breakdown of proopiomelanocortins (POMCs) into ACTH and β-endorphins (Itoi *et al*, 1994). Given TAAR1 regulation of monoamine activity, and monoamine release following MA exposure (Rothman *et al*, 2001), it is possible that MADR line differences in TAAR1 function, along with NET and SERT expression, regulate stress axis response to MA in these lines of mice.

MA appears to induce neural or physiological responses that are experienced as aversive in MALDR mice. The current results suggest that HPA axis activation may be one such unpleasant or aversive effect that contributes to

reduced MA intake. Sensitivity to the aversive effects of MA and MA intake corresponds with TAAR1 function in the MADR and *Taar1* transgenic mice, whereas there appears to be less correspondence of NET or SERT blockade with level of MA intake. Future investigation should make use of specific TAAR1 agonists to examine their ability to induce CTA, hypothermia, and CORT response, which may provide additional insight into the role of TAAR1 activation in these aversive responses to MA. It is possible that TAAR1 also limits MA intake in humans through increased sensitivity to aversive effects of MA. Therefore, pharmacotherapies designed to improve TAAR1 function may prove useful for reducing MA use or addiction.

CHAPTER 4: General Discussion

Goals and Hypotheses

MADR mice differ in their preference for MA drinking and total MA intake in a significant and reproducible way, based on consistent results in three replicate sets of MADR lines during selective breeding (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009). The goal of this dissertation was to explore the role of sensitivity to aversive effects of MA in MA consumption, using the MAHDR and MALDR lines and *Taar1* transgenic mice. I focused on aversive effects of MA, in part, because their role in addiction has been less studied than has the role of rewarding effects. Furthermore, high sensitivity to the aversive effects of MA has been consistently documented in MALDR mice at every MA dose tested (Shabani *et al*, 2012b; 2011; Wheeler *et al*, 2009), suggesting that it may play a protective role in risk for MA use. The consistent inverse relationship between MA drinking and sensitivity to MA-induced aversion indicates a significant genetic correlation between the two traits. I hypothesized that a gene or genes influencing sensitivity to MA-induced aversion also partially mediates reduced MA consumption through sensitivity to the aversive effects of MA.

In addition, some of my dissertation work focused on one gene candidate within the confidence interval of a QTL for MA consumption in MADR mice found on mouse chromosome 10 (Belknap *et al*, 2013). I speculated that *Taar1* is associated with sensitivity to the aversive effects of MA and thus, on MA consumption. In part, my decision to focus on this gene was based on previously reported agonist effects of MA at TAAR1, the role of TAAR1 in regulation of

monoamine transporter availability and disposition (Revel *et al*, 2011; Xie and Miller, 2008), the MADR line differences in NET and SERT expression (Wheeler *et al*, 2009), differences in the MADR line in the effects of MA on monoamine levels (Lominac *et al*, 2014), and a SNP found in *Taar1* of the MADR progenitor strains that predicts a conformational change in TAAR1 (Sanger Mouse Genome Project SNP Keane *et al*, 2011; Sanger, 2014; Yalcin *et al*, 2011).

I also tested the hypothesis that physiological effects of MA (i.e., body temperature and stress response) correspond with sensitivity to MA-induced aversion and intake in the MADR lines, such that high aversion and low intake are associated with larger effects on body temperature and a greater stress response. Based on my finding described in Chapters 2 & 3, and discussed below, I hypothesized that TAAR1 function is associated with these phenotypes, and that elimination of TAAR1 function would reduce sensitivity to MA-induced CTA. NET and SERT involvement in regulating the aversive effects of MA was also tested. I hypothesized that MALDR lines would be more sensitivity to the aversive effects of acute NET and SERT blockade, than MAHDR mice, and that NET and SERT blocker-induced CTA was mechanistically related to MA-induced CTA. Lastly, I hypothesized that increasing stress axis activity at the time that MA intake was being established would reduce MA intake in MAHDR mice.

Main Findings

Results from Chapters 2 and 3 are listed in Table 4.1. In the current experiments, it was found that the functional *Taar1* allele is dominant in its effect on MA intake, such that *Taar1* +/+ and +/- mice have generally low levels of

intake. Further, the segregation of the B6-like functional *Taar1* allele in MALDR mice confirms the direction of allele influence predicted by the QTL on chromosome 10 (Belknap *et al*, 2013), such that the B6-like allele was associated with lower MA intake in the QTL analysis. MA drinking, sensitivity to MA-induced CTA and thermal response to MA corresponded with *Taar1* genotype (Chapter 2). Homozygous expression of a non-functional D2 isoform of TAAR1 was associated with heightened genetic risk for MA intake (Chapter 2). Overall, these data provide strong support for *Taar1* as a candidate gene for regulation of MA consumption. In Chapter 3, I found that MA-induced elevation of plasma CORT corresponded with low MA intake and *Taar1* genotype. Only mice expressing a functional *Taar1* allele exhibited elevated CORT. Furthermore, consumption of CORT increased plasma CORT levels to a greater extent in mice also consuming MA, and also decreased preference for MA in MAHDR mice. These data support the hypothesis that elevated circulating CORT may be an aversive effect of MA that contributes to limiting MA consumption. Both MA-induced CORT elevation and low MA intake, are seen specifically in mice expressing functional *Taar1*. Finally, blockade of NET did not result in a differential conditioned aversive response in the MADR lines or alter MA-induced CTA, whereas results for SERT suggest a partial role in sensitivity to MA-induced aversion (Chapter 3).

Table 4.1: Summary of Current Results

Citation	Strain	Sex	Drug	Dose	Test	Result
Chapter 2	TAAR1 isoform cell cultures	-	MA	10^{-8} - 10^{-4} molar MA	cAMP accumulation	cAMP accumulated dose-dependently in cells expressing B6-like TAAR1, and was blocked by the TAAR1 antagonist, EPPTB. No cAMP accumulated in D2-like TAAR1 (non-functional) cells.
Chapter 2	MAHDR/ MALDR Rep 2	M/F	-	-	*Taar1* genotyping	MAHDR mice are homozygous for non-functional D2 allele. MALDR mice are homozygotes for functional B6 allele, or B6/D2 heterozygotes.
Chapter 2	MAHDR/ MALDR Rep 2 & 3, *Taar1*+/+, +/-, and -/-	M/F	MA	0, 1, 2, 4 8, or 16 mg/kg	Thermal response	MALDR, *Taar1*+/+ and +/- become hypothermic to low doses of MA. MAHDR and *Taar1* -/- become hyperthermic to low doses of MA.
Chapter 2	MAHDR/ MALDR Rep 2 *Taar1*+/+, +/-, and -/-	M/F	EtOH	2 or 4 g/kg	Thermal response	*Taar1* genotypes and MADR strains all developed dose dependent EtOH-induced hypothermia; no line or genotype difference.
Chapter 3	MAHDR/ MALDR Rep 2 & 3, *Taar1*+/+, +/-, and -/-	M/F	MA	0, 1, 2, or 4 mg/kg	HPA response	MA administration elevated plasma CORT in MALDR and *Taar1* +/+, but not MAHDR or *Taar1* -/- mice.
Chapter 2	*Taar1* +/+, +/-, and -/-	M/F	MA	0, 2 mg/kg	CTA	*Taar1* +/+ and +/- mice, but not *Taar1* -/- mice, were sensitive to MA-induced CTA.

Table 4.1: Summary of Current Results (continued)

Citation	Strain	Sex	Drug	Dose	Test	Result
Chapter 2	B6, D2, MAHDR/ MALDR Rep 2, *Taar1*+/+, +/-, and -/-	M/F	MA	20 or 40 mg/l	Two-bottle choice drinking	Mice with non-functional *Taar1* genotypes (MAHDR, D2, and *Taar1*-/-) consume high amounts of MA at 20 or 40 mg/l concentrations. Mice with functional *Taar1* genotypes (MALDR, B6, *Taar1*+/+, *and Taar1*+/-) consume low to no amounts of MA at either concentration.
Chapter 3	MAHDR Rep 2	M/F	CORT and MA	100 mg/l and 20 or 40 mg/l	Two-bottle choice drinking	CORT added to MA drinking solutions decreased preference for the MA-containing solution in MAHDR mice, and MA intake (mg/kg) only in male MAHDR mice.
Chapter 3	MAHDR/ MALDR Rep 2	M/F	NISX	0, 25, 50 mg/kg	CTA	Blockade of NET was sufficient to induce CTA in both MADR lines, but reduction in consumption did not differ between the lines.
Chapter 3	MAHDR/ MALDR Rep 2	M/F	FLUX	0, 12.5, 25, 50 mg/kg	CTA	CTA developed faster in MALDR compared to MAHDR mice at the lowest dose of the SERT-blocker tested, but blockade of SERT was sufficient to induce CTA in both MADR lines at all doses.
Chapter 3	MAHDR/ MALDR Rep 2	M/F	NISX	0, 50 mg/kg	NET blockade on MA-induced CTA	Repeated NET blockade did not alter the development of MA-induced CTA in MALDR mice.
Chapter 3	MAHDR/ MALDR Rep 2	M/F	FLUX	0, 50 mg/kg	SET blockade on MA-induced CTA	Repeated SERT blockade altered the development of MA-induced CTA in MALDR mice.

+/+, wildtype genotype; +/-, heterozygous genotype; -/-, knockout genotype; B6, C57BL6/J; cAMP, 3'-5'-cyclic adenosine monophosphate; CORT, corticosterone; CTA - conditioned taste aversion; D2, DBA/2J; EtOH, ethanol; EPPTB, N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide; F, female; FLUX, fluoxetine; HPA, hypothalamic-pituitary-

Table 4.1: Summary of Current Results (continued)

adrenal axis; kg, kilogram; l, liter; M, male; MA, methamphetamine; MADR, methamphetamine drinking mouse lines; MAHDR, methamphetamine high drinking mouse line; MALDR, methamphetamine low drinking mouse line; mg, milligram; mg/kg, milligram per kilogram; NET, norepinephrine transporter; NISX, nisoxetine; Rep, replicate; SERT, serotonin transporter; *Taar1*, gene encoding Trace amine-associated receptor 1; TAAR1, Trace amine-associated receptor 1

Response to Methamphetamine in MADR Mice

Published data from the MADR lines are summarized in Table 4.2. Although MADR mice have been used in numerous studies of MA intake and response, prior to this dissertation, studies have not focused on physiological responses to MA. Gene expression and measurement of neurotransmitter levels in response to MA have been investigated in the MAHDR and MALDR lines (Belknap *et al*, 2013; Lominac *et al*, 2014; Wheeler *et al*, 2009). Additionally, plasma MA levels following an acute injection of 2 mg/kg MA (Shabani *et al*, 2012b), and plasma MA levels following MA drinking (Eastwood *et al*, 2014) have been investigated in the MADR lines. Furthermore, extensive work has been done to catalog behavioral responses to MA and to investigate similarities in response to other drugs of abuse, notably cocaine and ethanol (see Table 4.2).

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Wheeler *et al*, 2009)	MAHDR/ MALDR Rep 1	M/F	MA, tastants	20 or 40 mg/l	Two-bottle choice drinking	MAHDR mice consumed about 7 mg MA/kg/18 h; MALDR mice consumed nearly none. Saccharin, KCl, and quinine tastant consumption was not different by line. Female MAHDR mice consumed more KCl than male MAHDR mice.
(Wheeler *et al*, 2009)	MAHDR/ MALDR Rep 1	M/F	MA	0.5 mg/kg	CPP	MAHDR mice exhibited MA-induced CPP (drug free), which was not seen in MALDR mice.
(Wheeler *et al*, 2009)	MAHDR/ MALDR Rep 1	M/F	MA	0, 1, 2 mg/kg	CTA	MALDR mice exhibited MA-induced CTA to both doses of MA, which was not seen in MAHDR mice.
(Wheeler *et al*, 2009)	MAHDR/ MALDR Rep 1	M	MA	0, 2 mg/kg	qPCR gene expression in NAcc	Naïve: Among other differences, MAHDR mice had lower *Htr3* and *Fos*, and higher *Slc6a4*, *Slc6a2*, and *Grm4* expression in the NAcc compared to MALDR mice. MA exposed: Increased expression of *Hsp40* and *Hsce70*, and decreased *Nfkb1* and *Nfkb2* in the NAcc did not differ between lines. Changes in expression following MA varied by gene and direction between the lines, but included decreased expression of *Mapk3* in MAHDR mice. Pathway analysis of Gene Ontology identified apoptotic and immune response pathways that were generally downregulated by MA in MALDR mice. These pathways were not generally regulated by MA in MAHDR mice, but genes involved in Toll-like receptor signaling were activated by MA. Pathways share genes such as *Nfkb2*, *Il6*, *Casp8,* and *Rela.*
(Shabani *et al*, 2011)	MAHDR/ MALDR Rep 2	M/F	MA, tastants	20 or 40 mg/l	Two-bottle choice drinking	MAHDR mice consumed about 6 mg MA/kg/18 h; MALDR mice consumed nearly none. Saccharin, KCl, and quinine tastant consumption not different by line.

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Shabani *et al*, 2011)	B6D2F2	M/F	MA	0.5, 1, 2, mg/kg	CPP	F2 mice exhibited MA-induced CPP (drug free) to 0.5 mg/kg MA, but not 1 or 2 mg/kg MA.
(Shabani *et al*, 2011)	B6D2F2	M/F	MA	0.5, 1, 2, mg/kg	Acute stimulation	F2 mice exhibited dose-dependent stimulation to MA.
(Shabani *et al*, 2011)	B6D2F2	M/F	MA	0.5, 1, 2, mg/kg	Locomotor sensitization	F2 mice exhibited dose-dependent MA-induced sensitization of locomotor stimulation that was significant for all doses of MA, but occurred more rapidly as dose increased.
(Shabani *et al*, 2011)	MAHDR/ MALDR Rep 2	M/F	MA	0.5, 1, 2, 4 mg/kg	CPP	MAHDR mice exhibited MA-induced CPP (drug free or present), which was not seen in MALDR mice.
(Shabani *et al*, 2011)	MAHDR/ MALDR Rep 2	M/F	MA	0.5, 2, 4 mg/kg	CPA	In the place preference test with drug present, MALDR mice exhibited MA-induced CPA that was not dose-dependent, an effect not seen in MAHDR mice.
(Shabani *et al*, 2011)	MAHDR/ MALDR Rep 2	M/F	MA	0.5, 2, 4 mg/kg	Acute stimulation	There was no line difference for acute locomotor stimulation to 0.5, 2, and 4 mg/kg MA.
(Shabani *et al*, 2011)	MAHDR/ MALDR Rep 2	M/F	MA	0.5, 2, 4 mg/kg	Locomotor sensitization	There was no line difference for MA-induced locomotor sensitization to 0.5 and 2 mg/kg MA; MAHDR mice exhibited MA-induced locomotor sensitization at 4 mg/kg MA that was not seen in MALDR mice.
(Shabani *et al*, 2012b)	B6D2F2	M/F	MA	0.5, 1, 2, 4, 8, 12 mg/kg	CPA	Mice developed a CPA (post-cue MA) that did not vary by MA dose.
(Shabani *et al*, 2012b)	MAHDR/ MALDR Rep 2	M/F	MA	0.5, 2, 4 mg/kg	CPA	MAHDR mice exhibited CPA (post-cue MA) after conditioning with 4 mg/kg MA; MALDR mice exhibited robust CPA at 2 and 4 mg/kg MA, but not 0.5 mg/kg.

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Shabani *et al*, 2012b)	MAHDR/ MALDR Rep 2	M/F	MA	MALDR= 0, 1, 2 mg/kg; MAHDR = 0, 2, 4 mg/kg	CTA	MAHDR mice did not develop MA-induced CTA; MALDR mice exhibited robust MA-induced CTA at both doses.
(Shabani *et al*, 2012b)	MAHDR/ MALDR Rep 2	M/F	MA	2 mg/kg	MA metabolism	Both lines reached peak MA plasma levels at 15 minutes following i.p. MA treatment; significantly higher plasma MA levels were found in MAHDR, than MALDR, mice at 15 minutes following administration, but the lines do not differ at other time points (0, 30, 60, 120, or 240 minutes).
(Shabani *et al*, 2012a)	MAHDR/ MALDR Rep 2	F	MA	40 mg/l	SA	MAHDR mice exhibited more robust operant oral MA SA, compared to MALDR mice, as measured by active lever presses and MA intake.
(Shabani *et al*, 2012a)	MAHDR/ MALDR Rep 2	M	MA	0, 0.1, 0.5, 1, 2.5, 5 µg/inf	SA	MAHDR mice exhibited more robust intracranial MA SA, compared to MALDR mice, as measured by MA infusions.
(Eastwood and Phillips, 2012)	MAHDR/ MALDR Rep 2	M/F	Fentanyl	0.05, 0.1, 0.2, 0.4 mg/kg	Hot plate, Tail flick	There were no line differences in sensitivity to the analgesic effects of fentanyl analgesia.
(Eastwood and Phillips, 2012)	MAHDR/ MALDR Rep 2	M/F	Fentanyl	0.05, 0.1, 0.2, 0.4 mg/kg	Acute stimulation	MALDR mice exhibited greater locomotor stimulation to fentanyl.
(Eastwood and Phillips, 2012)	MAHDR/ MALDR Rep 2	M/F	Morphine	10, 20, 30 mg/kg	Acute stimulation	MALDR mice exhibited greater locomotor stimulation to morphine.
(Eastwood and Phillips, 2012)	D2/B6	M/F	MA	20, 40 mg/l	Two-bottle choice drinking	D2 mice consumed more MA (about 4.5 mg/kg/18 h) than B6 mice (about 0.5 mg/kg/18 h).

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Moschak *et al*, 2012)	MAHDR/ MALDR Rep 2	M/F	MA	0, 0.5, 1, 2 mg/kg	Go/no go task	There was no line difference in behavioral inhibition at baseline or after MA treatment. Hits were initially higher in MA-naïve MALDR mice. MA decreased false alarms, pre-cue responses, and hits in both lines, with some resistance to MA effects on hits in female MAHDR mice.
(Belknap *et al*, 2013)	MAHDR/ MALDR Rep 1 & 2	M/F	-	-	QTL	A QTL on chromosome 10 between 10 and 40 Mb accounts for ~50% of the genetic variance in MA drinking in MADR mice. D2 alleles in this region are associated with higher drinking.
(Belknap *et al*, 2013)	MAHDR/ MALDR Rep 1	M/F	-	-	Gene expression	There were many differentially expressed genes between the MADR lines in the NAcc, PFC, and VMB. Within the chromosome 10 QTL confidence interval, there were 12 in the NAcc, 30 in the PFC, and 20 in the VMB. Of particular interest within these areas were several genes that were differentially expressed, including: *Oprm1*, *Esr1*, *Hivep2*, and *Map3k5*. *Taar1* was included in the analysis but was not differentially expressed between the MADR lines.
(Olsen *et al*, 2013)	MAHDR/ MALDR Rep 2	M/F	MA	-	Behavioral testing	*Open field*: there were no line differences in baseline activity. *Elevated zero maze*: there were no line differences in total distance or metrics of anxiety-like behavior. *Novel object:* there were no line differences in total exploration or time exploring a novel object. *Water maze:* the only significant difference was for spatial retention memory for the target on probe trials, for which MAHDR mice exhibited no evidence for memory, but MALDR mice did. *Fear conditioning:* There were no line differences.
(Olsen *et al*, 2013)	MAHDR/ MALDR Rep 2	M/F	MA	0, 25, 50 mg/l	Circadian rhythm	There was no line difference in free-running period (τ) during access to water. During access to 25 mg/l MA, τ was increased only in MALDR mice; during access to 50 mg/l, τ was increased, and there was no line difference. There was a positive correlation of MA intake and τ in MALDR mice only.

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Gubner *et al*, 2013)	MAHDR/ MALDR Rep 2	M/F	Cocaine	10 mg/kg	CPP	There was significant cocaine-induced CPP in both the drug-free and drug-present tests and cocaine-induced stimulation during the drug-present test, but the lines did not differ.
(Gubner *et al*, 2013)	MAHDR/ MALDR Rep 2	M/F	Cocaine	15, 30 mg/kg	CTA	There was significant cocaine-induced CTA at both doses, but the lines did not differ.
(Gubner *et al*, 2013)	MAHDR/ MALDR Rep 2	M/F	Cocaine	5, 10, 20, 30 mg/kg	Acute stimulation	There was significant, dose-dependent locomotor stimulation, but the lines did not differ.
(Eastwood *et al*, 2014)	MAHDR/ MALDR Rep 2	F	MA	20, 40, 80 mg/l	Lickometer	Both lines consumed about 3.2 mg MA/kg/4 h on the first day of access to the 20 mg/l MA concentration. MALDR mice decreased and MAHDR mice increased consumption of MA on all subsequent sessions, and at other concentrations.
(Eastwood *et al*, 2014)	MAHDR/ MALDR Rep 2	F	MA	20, 40 mg/l	Plasma MA levels following drinking	Blood MA levels were similar in MADR lines following first drinking session. MAHDR mice had higher MA levels after subsequent drinking sessions. Plasma levels corresponded with amount of MA consumed.
(Eastwood and Phillips, 2014)	MAHDR/ MALDR Rep 2	M/F	Morphine	.3, .7, 1 mg/ml	Two-bottle choice drinking	MALDR mice consumed more morphine than MAHDR mice.
(Eastwood and Phillips, 2014)	MAHDR/ MALDR Rep 2	M/F	μ-receptor agonist or antagonist	20, 40, 80 mg/l MA	Two-bottle choice drinking	Naltrexone (μ antagonist) pretreatment (0, 0.5, 1, 2, 5, 10, or 20 mg/kg) had no effect on MA consumption. Buprenorphine (μ-receptor partial agonist) pretreatment (1 or 2 mg/kg, but not 0, or 4 mg/kg) reduced MA intake in MAHDR mice and had no effect on MA intake in MALDR mice.
(Lominac *et al*, 2014)	B6	M	MA	0, 2 mg/kg	Conventional microdialysis	Acute MA increased extracellular DA in NAcc and mPFC. 10 daily MA injections prior to testing increased DA concentrations in both brain regions, and a 21-day period of withdrawal, further increased DA concentrations.

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

Citation	Strain/Line	Sex	Drug	Dose	Test	Result
(Lominac *et al*, 2014)	B6	M	MA	0, 2 mg/kg	CPP	B6 mice developed MA-induced CPP. Intra-NAcc infusion of the DAT inhibitor GBR12909 (100nM) prior to testing, increased CPP expression. Intra-NAcc infusion of the D_2/D_3 agonist quinpirole (100nM) prior to testing, induced CPA. Intra-mPFC infusion of either drug had no effect.
(Lominac *et al*, 2014)	MAHDR/ MALDR Rep 2	F	MA	0, 2 mg/kg	Basal transmitter-level microdialysis (No net-flux)	MAHDR mice had lower basal levels of DA in the NAcc and mPFC. MAHDR had higher 5-HT in the NAcc, but there was no line difference in the mPFC for 5-HT. MA administration increased DA and decreased 5-HT in the mPFC in MAHDR mice. Conversely, MA decreased DA and increased 5-HT in the mPFC in MALDR mice. No differences were seen in the NAcc.
(Lominac *et al*, 2014)	MAHDR/ MALDR Rep 2	F	MA	0, 2 mg/kg	Monoamine transporter protein expression	NAcc shell: MAHDR had higher expression of DAT and SERT, but lower expression of D_2Rs than MALDR mice. NAcc core: MAHDR had higher expression of DAT, but lower expression of 5-HT1B, than MALDR mice. mPFC: MAHDR had a trend toward higher 5-HT1B expression than MALDR mice.

5-HT, serotonin; 5-HT1B, serotonin 1B receptor; B6, C57BL6/J; B6D2F2, C57BL/6J x DBA/2J F2 cross; *Casp8,* caspase 8, apoptosis-related cysteine peptidase gene; CPA, conditioned place aversion; CPP, conditioned place preference; CTA - conditioned taste aversion; DA, dopamine; DAT, dopamine transporter; D_2Rs, dopamine D_2 receptors; D_2/D_3, dopamine D_2 and D_3 receptor; D2, DBA/2J; *Esr1*, estrogen receptor 1 gene; F, female; F2, second filial generation offspring; *Fos*, FOS gene; *Grm4,* metabotropic glutamate receptor 4 gene; *Hivep2*, human immunodeficiency virus type 1 enhancer

Table 4.2 Summary of Previous Findings in MADR Lines and Progenitor Strains (continued)

binding protein 2 gene; h, hour; *Hsce70*, immunoglobulin heavy chain binding protein 70 gene; *Hsp40*, heat shock protein 40 gene; *Htr3*, serotonin receptor 3 gene; *Il6*, interleukin 6 gene; inf, infusion; i.p., intraperitoneal; KCl, potassium chloride; kg, kilogram; l, liter; M, male; MA, methamphetamine; MADR, methamphetamine drinking mouse lines; MAHDR, methamphetamine high drinking mouse line; MALDR, methamphetamine low drinking mouse line; *Map3k5*, mitogen-activated protein kinase kinase kinase 5; *Mapk3*, ERK-1 MAP kinase gene; Mb, megabase; µg, microgram; Mg, milligram; mg/kg, milligram per kilogram; mPFC, medial prefrontal cortex; NAcc, nucleus accumbens; *Nfkb1,* nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 gene; *Nfkb2*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 gene; nM, nanomolar; *Oprm1*, µ opioid receptor gene; PFC, prefrontal cortex; qPCR, quantitative polymerase chain reaction; QTL, quantitative trait locus; *Rela*, NFκB subunit p65 gene; Rep, replicate; SA, self-administration; SERT, serotonin transporter; *Slc6a2,* NET gene; *Slc6a4*, SERT gene; (*T)*, circadian free-running period; VMB, ventral midbrain.

Methamphetamine Consumption

Divergence in MA consumption by the MADR lines has been published for each of the three replicate selections that were completed before or during the time that this dissertation work was being performed (Harkness *et al*, 2015; Shabani *et al*, 2011; Wheeler *et al*, 2009). Each of these selections produced remarkably similar results for MA consumption, with MAHDR mice consuming between 6 and 7 mg MA/kg/18 h, and MALDR mice consuming about 0.5 mg MA/kg/18 h on average by the last generation of selection. However, wide variation in MA consumption appears to exist in MAHDR mice, even after multiple generations of selection, at which time some individuals consume very large amounts of MA, whereas others consume relatively little MA. MALDR mice, on the other hand, show less variation at the end of selection, with all individuals consuming low amounts of MA. For example, MAHDR mice from replicates 1-3 in the fourth generation of selection ranged in consumption from 0.0-6.9 mg/kg MA at the 20 mg/l concentration and 0.0-12.0 mg/kg MA at the 40 mg/l concentration. On the other hand, MALDR mice ranged in consumption from 0.0-1.4 mg/kg MA at the 20 mg/l concentration and 0.0-1.7 mg/kg MA at the 40 mg/l concentration (Phillips lab; unpublished observation).

These observations raise the possibility that a mechanism responsible for preventing MA consumption in the MALDR line, perhaps TAAR1 function, is common to all mice of the MALDR genotype and dominant over mechanisms that may influence MA intake. I hypothesize that the absence of TAAR1-mediated inhibition of MA consumption results in varying MA intake in MAHDR mice due to

the influence of other genetic or nongenetic factors. Our data suggest that homozygous or heterozygous expression of functional TAAR1 is sufficient to protect against MA consumption. In the MADR lines, the B6 *Taar1* allele is the functional form and has a dominant influence on MA intake. Although our data thus far indicate that all MALDR mice are either homozygous or heterozygous for the B6 allele and that MAHDR mice are homozygous for the alternative D2 allele, which encodes a non-functional TAAR1, we have sequenced the relevant polymorphism in relatively few mice (Harkness *et al*, 2015). Additional genotyping is underway to further examine the relationship between this polymorphism and MA intake.

Taste Perception in Methamphetamine Consumption

Evidence that I presented in Chapters 2 and 3 supports TAAR1 function-related regulation of MA consumption in the MADR lines, and I have hypothesized that TAAR1 regulation of monoamine system function is associated with sensitivity to the negative effects of MA. However, it is possible that indirect selection of a co-varying trait or unintended trait can explain part or all of the selected phenotype (Falconer and MacKay, 1996). For example, MA is reported to have a bitter taste (NIDA, 2015). It is possible that diverging MA intake in the MADR lines is due to sensitivity or insensitivity of bitter taste perception, rather than sensitivity to the pharmacological effects of MA. Therefore, the MADR lines have been tested for consumption of psychoactively inert tastants including, sweet, salty, and bitter flavors (Shabani *et al*, 2011; Wheeler *et al*, 2009). Overall, the lines have not been found to differ in consumption of tastants. Wheeler *et al.*

(2009) reported a significant sex x line x concentration interaction for level of consumption of salty KCL solution in the first replicate MADR lines. Further examination revealed that female MAHDR mice consumed more KCl than male MAHDR mice, but that there was no significant difference in consumption between the lines. Similarly, a significant sex x line x concentration interaction was found for KCL consumption in the second replicate MADR selection (Shabani *et al*, 2011). However, further statistical examination of this interation failed to identify any significant sex or line differences.

Eastwood and Phillips (2012) reported that MAHDR mice consumed a significantly greater amount of quinine solution in a two-bottle choice experiment. However, in this experiment, quinine was offered as an alternative to morphine, a solution that was largely avoided by MAHDR mice and consumed by MALDR mice. Therefore, in this case, the line difference in quinine consumption was attributed to MAHDR avoidance of morphine rather than seeking quinine. This conclusion was supported by similarity in total volume of fluid consumed between the lines, indicating that regulation of fluid consumption likely accounted for the line difference in quinine intake (Eastwood and Phillips, 2012).

Additional evidence that the line difference in MA consumption is not simply due to a difference in taste sensitivity comes from operant SA studies. Similar to two-bottle choice consumption, the lines differed in intracranial and oral SA of MA (Shabani *et al*, 2012a). MAHDR mice will self-administer intracranial or oral MA; however, the MALDR line will not self-administer either. SA of intracranial MA entirely bypasses taste factors, which provides additional support

for the conclusion that differences in MA consumption between the lines cannot be simply explained by taste perception. Low responding for MA by MALDR mice in both intracranial and oral SA procedures suggests that low responsivity is dependent on MA effects that would be experienced in both procedures, such as aversive pharmacological effects of MA.

Additional evidence for this conclusion includes that MALDR mice drink MA at levels identical to MAHDR mice during the first session that the MA solution is offered, but consume little to no MA following this initial experience (Eastwood *et al*, 2014; Shabani *et al*, 2012a). This suggests that low intake in MALDR mice is not due to aversive factors readily apparent upon the first taste of MA. Rather, it is a learned aversion delayed by a period of time, possibly allowing for pharmacological effects to be experienced. Initial levels of MA consumption by MALDR mice indicates that these mice do not avoid MA because of a preexisting distaste for the flavor; further evidence that taste perception of MA does not explain MADR line differences in MA drinking. Therefore, perception of solution taste is not considered to be a factor in differential MA consumption between the MADR lines, and line differences in MA consumption likely result from pharmacological differences.

Cognitive, Impulsivity, and Anxiety-like Traits in Methamphetamine Consumption

Acute and repeated MA have anxiogenic effects in mice (Hayase *et al*, 2005). Impulsivity, anxiety-like behavior, or fear of novel objects could impact MA intake as a correlate of selection, by reducing exploratory or approach behavior when MA is introduced. For example, elevated anxiety-like behavior could reduce

readiness to drink novel MA solutions. Olsen *et al*. (2013) tested the MADR lines for several measures of anxiety-like behavior. MA-naïve MADR mice did not differ by line in elevated-zero maze performance, novel object exploratory behavior, or fear conditioning behavior. Mice were also tested for shifts in free running period with access to MA as a measure of sensitivity to MA-induced shifts in circadian rhythm. There were no differences between MA-naïve MAHDR and MALDR mice in free-running period (τ). MALDR mice exhibit an increased τ during access to 25 mg/l MA, an effect not found in MAHDR mice. Access to 50 mg/l MA increased τ in both lines to a similar extent. MA intake correlated positively with τ in the MALDR mice only. These results suggest greater sensitivity to MA-induced shifts in circadian rhythm in MALDR mice. It is possible that high sensitivity to shifts in circadian rhythm are aversive to the MALDR mice, and lead to a reduction in MA intake. However, measures of anxiety did not differ between the lines and thus may not mediate sensitivity to rewarding or aversive effects of MA.

Learning ability and capacity to remember the rewarding or aversive effects of MA could be another determining factor in MA intake between the lines. Attenuated cognitive ability could reduce learned associations between MA exposure and the aversive effects of MA. The lines were found to significantly differ in MA-naïve retention of learned platform position during testing in the Morris water maze. While there were no line differences in velocity or latency to platform on trial days or quadrant bias on probe days, MAHDR had significantly reduced spatial retention memory for the target on probe trials compared to

MALDR mice (Olsen *et al*, 2013). This suggests that the MAHDR line may have deficits in hippocampal-dependent learning. Olsen *et al.* (2013) proposed that altered expression patterns in AMPA subunit expression could underlie this deficit, which was previously associated with impairments in cognition, working spatial memory, and increased MA exposure (Simoes *et al*, 2007).

Overall, the lines do not differ in measures of impulsivity, anxiety-like behavior, or fear of novel objects – which could be relevant to initial exposure to a tube of MA solution, suggesting that line differences in MA consumption are not due to behavioral tendencies not directly related to MA intake. However, lower performance by the MAHDR line in cognitive and/or memory measures could potentially impact learning of MA effects. If attenuated hippocampal dependent learning in the MAHDR line were indicative of greater cognitive deficits, it might be possible that the MAHDR line is simply not learning that MA is aversive. However, it would then be expected that MAHDR mice would not be sensitive to conditioned aversion induced by other drugs, yet cocaine induced a strong CTA and there was no difference in sensitivity to cocaine-induced CTA between the MAHDR and MALDR lines (Gubner *et al*, 2013). In addition, cocaine induced significant CPP in both drug-free and drug-present tests, and the lines did not differ in this response (Gubner *et al*, 2013). Rather, MADR line differences in rewarding and aversive responses to MA are consistent across varying procedures and doses, and do not extend to another psychostimulant drug. Thus, while cognitive performance may segregate with MA intake, the connection between MA preference and cognitive performance is not clear. It is also possible

that cognitive performance is a correlated response resulting from selection-trait irrelevant gene fixation that arose as the result of inbreeding during selection (Crabbe J., 1999; Palmer A. A. & Phillips T. J., 2002). Olsen *et al.* (2013) used mice from the fifth generation of selection of the second replicate set of lines. Duplication of the Morris water maze experiment with other replicates of MADR mice would help to determine if this association is consistently found and a genetic correlation reflecting phenotypic effects of gene association with MA intake.

Methamphetamine Reward

MADR mice differ in MA consumption likely due to differences in sensitivity to both rewarding and aversive effects of MA. The MAHDR line has consistently been found to be more sensitive to the rewarding effects of MA in measures of CPP. Wheeler *et al.* (2009) first demonstrated this line difference for a 0.5 mg/kg dose of MA, using the drug-absent procedure. While the MAHDR line showed significant CPP, the MALDR line did not. However, MALDR mice did not show significant preference for either the paired or unpaired floor, indicating that they conditioned neither a preference nor an aversion to MA in this procedure. Shabani *et al.* (2011) again tested CPP in the MADR lines, using mice from the second replicate of selection. This time, place preference was tested under both drug-absent and drug-present conditions across a range of doses. Again, the drug-absent test of CPP resulted in a line difference, wherein MAHDR mice exhibited CPP to the MA-paired context that did not differ by MA dose, but MALDR mice did not demonstrate preference or aversion (Shabani *et*

al, 2011).

The drug-present test, however, resulted in CPA in the MALDR line to the MA-paired context, but CPP in the high line at only 0.5 mg/kg. Although, when corrected for locomotor behavior, preference for the MA-paired floor in MAHDR mice was also found at 2 mg/kg when MA was present; a covariate that is discussed in detail below. This is, while the MALDR line did not demonstrate CPA to MA in the drug-absent place preference test, the same doses of MA did produced CPA when drug is present. D2 mice, the progenitor strain that most resembles MAHDR mice for MA drinking, induce CPP or CPA depending on MA dose in the CPP test (Cunningham and Noble, 1992). In a drug-absent test, these mice were found to be sensitive to CPP at 0.5 mg/kg MA, non-preferring at 8 mg/kg, and exhibited CPA at 16 mg/kg MA. However, in a drug-present test, 8 mg/kg MA produced CPA. Thus, drug-present place preference testing appears to be more sensitive at detecting conditioned aversive effects of MA. Cunningham and Noble (1992) suggest that the drug-present and drug-absent place preference procedures expose bivalent rewarding and aversive effects of MA at the same dose either because of interoceptive processes similar to state-dependent learning, or because drug being present increased the expression of aversive hedonic values conditioned to the paired floor (Cunningham and Noble, 1992).

MA reward has also been investigated in the MADR lines using oral and intracranial SA procedures, which have demonstrated MA seeking by MAHDR, but not MALDR mice (Shabani *et al*, 2012a). These results again demonstrate

that the MAHDR line is highly sensitive to the rewarding properties of MA, which is not seen in the MALDR line. This conclusion is strengthened by similarity in line differences from multiple modalities of testing MA reward (CPP and SA). Pretreatment with a TAAR1 agonist reduce operant SA of MA in rats (Jing *et al*, 2015), indicating that TAAR1 may be involved in motivational elements of MA intake. The authors note that the TAAR1 agonist appears to specifically reduce MA cue-related motivational properties, rather than suppress general motivational states of the rodent (Jing *et al*, 2015).

An additional mechanism in MA reward processing may be the opioid system. In the MADR lines, studies pertaining to the μ-opioid receptor gene, *Oprm1*, were initiated because it is located on chromosome 10 in the vicinity of the large MA drinking QTL. The MALDR line consumes more morphine than the MAHDR line (Eastwood and Phillips, 2014), and this could suggest a role for the opioid system in the rewarding effects of MA. MA dependence and psychosis in humans are associated with polymorphisms and linkage disequilibrium in the gene encoding the μ-opioid receptor, *OPRM1* (Ide *et al*, 2006). Additionally, a μ-opioid receptor partial agonist, buprenorphine, reduced DA activity following administration of MA to rats (Pereira *et al*, 2011) and also reduced MA intake in MAHDR mice (Eastwood and Phillips, 2014). This suggests that MA-induced monoamine activity could be partially regulated by opioid systems, and that higher MA intake may be associated with lower sensitivity to μ-opioid receptor agonists. In fact, MAHDR mice are less sensitive to opioid locomotor stimulation

than MALDR mice, indicating greater opioid sensitivity in MALDR mice (Eastwood and Phillips, 2012).

These data suggest that heightening opioid system activity might be an effective way of reducing MA intake. In the study by Eastwood and colleagues, pretreatment of MAHDR mice with 1 or 2 mg/kg, but not 4 mg/kg, of the partial agonist buprenorphine, reduced MA intake (Eastwood and Phillips, 2014). It was speculated that the 4 mg/kg dose was not effective because such higher doses of buprenorphine have μ-opioid receptor antagonist-like effects. Furthermore, pretreatment with naltrexone (a μ-opioid receptor antagonist) at doses ranging from 0.5 to 20 mg/kg, had no effect on MA consumption (Eastwood and Phillips, 2012). Overall, these experiments indicate that reduced opioid sensitivity and low morphine consumption segregate with selection for high MA intake in the MADR lines. Thus, increased opioid sensitivity may be genetically linked to low MA intake and high aversion for MA.

It is possible that activation of opioid pathways interacts with monoamine systems to mediate some effects of MA. For example, locomotor stimulation by opioids may result from DAergic activity (Eastwood and Phillips, 2012). It is also possible that other genes in the MA-intake network that have more direct effects on MA intake regulate *Oprm1*. A network analysis of risk for MA intake identified *Oprm1* as a significant hub regulated by nine of the top ranking transcripts that were differentially expressed in brain tissue from the MADR lines in the MA-naïve state (Belknap *et al*, 2013). Thus, *Oprm1* may be a downstream target, rather than a predictor of risk for MA use.

Methamphetamine Stimulation

Consumption of a drug solution can be affected by sensitivity to drug effects. Acute or sensitized stimulation to MA has been investigated in the MADR lines (Eastwood and Phillips, 2012; Olsen *et al*, 2013; Shabani *et al*, 2011), as well as for cocaine (Gubner *et al*, 2013). The lines have been found to be largely similar in their locomotor response to several low to moderate doses of these drugs (Table 4.2). There is one example, however, of greater locomotor sensitization in the MAHDR line to repeated 4 mg/kg MA (Shabani *et al*, 2011). Here, mice were injected with MA prior to CPP conditioning over 6 trials and monitored for locomotor behavior. In a drug-present preference test, MA did not appear to induce CPP at this dose, (Shabani *et al*, 2011). Shabani *et al*. (2011) proposed that locomotor activity interfered with the expression of CPP at this dose, because high locomotor activity has been associated with weaker CPP (Cunningham *et al*, 2006a). Differences in locomotor behavior can be controlled for during statistical analysis of place preference data by covarying for distance traveled (Cunningham *et al*, 2006a; Shabani *et al*, 2011). As mentioned above, place preference for 2 mg/kg MA, when MA was present, was only statistically significant in MAHDR mice when locomotor activity was included as a covariate in the analysis (Shabani *et al*, 2011).

MA-induced stereotypy, which could also interfere with locomotor behavior, is not likely related to the difference in sensitization, as it has not been found to differ between the lines (Phillips unpublished data). However, the difference in MA-induced locomotor sensitization at this dose may indicate

underlying differences in neuroadaptation (Phillips *et al*, 2008). Additionally, greater acute locomotor stimulation to amphetamine and MA have been reported in *Taar1* -/- mice compared to their WT counterparts (Achat-Mendes *et al*, 2012; Lindemann *et al*, 2008; Wolinsky *et al*, 2007). We have some evidence for this in unpublished data from multiple replicates of the MADR lines, with MAHDR mice showing greater stimulation than MALDR mice in some studies, particularly at higher doses. Thus, differences in MA-induced locomotor stimulation and sensitization could be additional behavioral effects associated with *Taar1* genotype in *Taar1* transgenic and MADR mice.

The potential relationship between locomotor effects of MA and MA consumption needs further exploration. There is some evidence for a relationship between ethanol consumption and sensitization (Lessov *et al*, 2001). For example, B6 mice drink large quantities of ethanol, but are insensitive to sensitization of the stimulating effects of ethanol, characteristics that are the opposite of D2 mouse sensitivities to ethanol (Cunningham *et al*, 1992; Phillips *et al*, 1994; Phillips *et al*, 1996). However, this 2-strain comparison does not allow one to draw genetic conclusions. On the other hand, mice derived from a cross of two selectively bred lines for High Alcohol Preference (cHAP) will consume alcohol to intoxication, but do not develop locomotor sensitization to alcohol (Matson *et al*, 2014), supporting the relationship that has been seen in the B6 and D2 mice. However, mice selected for high alcohol locomotor stimulation (FAST) consumed greater amounts of alcohol than mice selected for low alcohol sensitivity (SLOW), suggesting that the relationship between consumption and

sensitivity is not the same as between consumption and acute stimulation (Risinger *et al*, 1994).

In the MAHDR line, propensity for MA-induced locomotor sensitization is related to a preference for MA consumption, but the basis of this relationship is not clear. One hypothesis is that greater sensitization reflects greater neuroadaptation in neurochemical systems associated with avidity for MA. However, a negative genetic correlation between MA sensitization and intake has been described for MA in lines that were selectively bred for degree of MA-induced sensitization. The MA sensitization lines or MAHSENS and MALSENS (described in Chapter 1), differ not only in degree of sensitization, but also in acute stimulation to MA and MA intake. MAHSENS mice, bred for high MA sensitization, exhibited greater stimulation and, initially, greater MA intake than MALSENS mice (Scibelli *et al*, 2011). However, after additional generations of selection, the line difference in MA intake was reversed, so that MALSENS mice exhibited greater MA intake. This led the authors to suggest that extreme sensitization, as might be induced by selection for this trait, may be an aversive experience and result in reduced MA intake (Scibelli *et al*, 2011). Because the line difference in sensitization in the MADR lines occurs only at higher MA doses, they may not exhibit the more extreme sensitization that may have been induced by selection for this trait using a 1 mg/kg dose of MA.

Methamphetamine Aversion

The effectiveness of the CTA procedure relies on pairing the perception of an aversive effect with a taste (Cappell H. & LeBlanc A. E., 1971). In the case of

the two-bottle choice procedure, pairing of taste with an aversive effect protects the animal from consumption of an aversive solution, and leads to learned solution preference. Meisch *et al*. (2001) describe that tastes can become conditioned stimuli to a rodent during a drinking procedure. I hypothesized that a similar process influences MA drinking in MALDR mice. MALDR mice are highly sensitive to the conditioned aversive effects of MA (Shabani *et al*, 2012b; 2011; Wheeler *et al*, 2009). A similar conditioned effect could occur during 2-bottle choice drinking. Essential evidence in favor of my hypothesis is that MALDR mice drink MA at the same level of MAHDR mice during the first session that the MA solution is offered, but consume little to no MA following this initial experience (Eastwood *et al*, 2014; Shabani *et al*, 2012a). This suggests that MA aversion in MALDR mice is dependent on the outcome of the first experience with MA. That is, low intake is not due to aversive factors readily apparent upon the first taste of MA, but rather is learned aversion, delayed by some short period of time, possibly requiring pharmacological effects to be experienced.

As described in Chapter 1, MA consumed orally does not reach peak plasma concentration in humans for 2.5-7.5 h after intake (Cook *et al*, 1992; Schepers *et al*, 2003; Shappell *et al*, 1996). In the MALDR mice, the pharmacological delay may allow significant consumption before the mice begin to experience the aversive effects of MA. However, blood MA data suggests that peak plasma levels occur as early as 15 min after i.p. administration in our mice (Shabani *et al*, 2012b). As presented in Chapters 2 and 3 and discussed in detail below, I studied two effects of MA that may be subjectively aversive: hypothermia

and increased plasma CORT. These effects reach peak levels 15-30 minutes following an acute injection of MA. Oral MA intake may prolong the onset of peak hypothermia or HPA response, which could be experienced during the first period of access to MA in MALDR mice. More investigation is needed to determine if HPA response and hypothermia response occur following oral MA intake on a time scale that corresponds to MA consumption during the first 1-h or 2-h bin measured by Shabani et al, (2012a) and Eastwood *et al.* (2014). Associating the unconditioned aversive effects of MA with MA intake may result in decreased MA intake in MALDR mice; an effect not unlike a self-imposed CTA to MA. MAHDR mice would not be expected to experience this process based on their insensitivity to MA-induced CTA up to acute doses of 4 mg/kg, the highest dose we have tested (Shabani *et al*, 2012b).

TAAR1-Associated Methamphetamine Effects

Thermal Response

Heightened sensitivity to MA-induced hypothermia is associated with low MA intake and greater sensitivity to MA-conditioned aversion (Chapter 2). Because MA is an agonist at TAAR1 (Bunzow *et al*, 2001; Reese *et al*, 2014; Wolinsky *et al*, 2007), I examined the literature and found reports of TAAR1 agonist-induced hypothermia in rodents (Di Cara *et al*, 2011; Fantegrossi *et al*, 2013; Panas *et al*, 2010; Sabol *et al*, 2013). In addition, my data in *Taar1* transgenic mice supported a role for TAAR1 in MA-induced hypothermia. Thus, it appears that TAAR1 activation has a role in MA-induced hypothermia and that

the immediate hypothermic effect of MA may play a role in curbing MA intake in MALDR, *Taar1* +/+ and *Taar1* +/- mice.

This hypothesis could be tested by preventing the hypothermic effect in MALDR mice, while measuring MA intake. Operant SA of both MA (Cornish *et al*, 2008) and MDMA (Cornish *et al*, 2003) was elevated by an increase in ambient temperature to 30°C, relative to normal temperature of 21-23°C. This suggests that high ambient temperature increases the rewarding effects of these drugs. However, reduced body temperature alone does not induce CTA in rodents (Misanin *et al*, 1998), although hypothermia did prolong the associative period during which aversion could be conditioned (Christianson *et al*, 2005; Misanin *et al*, 1998; 2002). This suggests that hypothermia alone is not responsible for MA aversion in the MALDR line, but that MA-induced hypothermia may intensify the association of MA with unpleasant physiological or subjective effects of MA.

Sensitivity to ethanol-induced hypothermia appears to be regulated by genetic factors distinct from those that influence sensitivity to the thermal effects of MA, as the response to ethanol was similar in both the transgenic and MADR mice. Doses of ethanol (2 or 4 g/kg) known to induce hypothermia in mice were used (Crabbe *et al*, 1979). In mouse lines selected for HOT and COLD response to ethanol, a line difference in 5-HT activity was implicated as one mediator of differing ethanol-induced hypothermic response (Feller *et al*, 1993). The MADR lines do differ in SERT expression and basal 5-HT activity in the NAcc (Lominac *et al*, 2014). However, given that the MADR lines do not differ in their hypothermic response to ethanol, it seems likely that ethanol hypothermia is not

mediated by these particular differences. Furthermore, unlike MA, there is no evidence for direct or indirect agonist activity of ethanol at TAAR1.

Stress Axis Response to Methamphetamine

Plasma ACTH and CORT were elevated in MALDR, but not MAHDR, mice following MA treatment (Fig. 3.1). CORT levels were also elevated by MA treatment in *Taar1* +/+, but not -/-, mice (Fig 3.2). Data for the effect of MA on CORT were consistent in two independent sets of the MADR lines, and elevated CORT corresponded with the *Taar1* polymorphism that impacts TAAR1 function. These are the first data to suggest that TAAR1 function may affect HPA response to MA, although it was previously reported that intracerebral and systemic administration of two thyronamine compounds (T_1AM and T_0AM), with agonist actions at TAAR1, increased plasma CORT levels in rats (Klieverik *et al*, 2009). The effect of MA on CORT levels in MALDR mice was somewhat larger than in *Taar1* +/+ mice, suggesting that selection for low MA intake may have altered additional genetic mechanisms relevant to regulation of HPA axis response to MA.

In accordance with my hypothesis that elevated plasma CORT response may be an aversive effect of MA, the addition of CORT to the MA drinking solution decreased the preference for the MA-containing solution in MAHDR mice. As discussed in Chapter 3, elevated plasma CORT may be important to learning the aversive properties of MA during the initial exposure(s) to MA. MAHDR mice previously exposed to MA in the absence of CORT do not reduce

consumption when CORT is added to the MA solution. However, when CORT is in solution with MA during initial access, MA consumption decreases over time.

Given TAAR1 regulation of monoamine activity, and monoamine release following MA exposure (Rothman *et al*, 2001), it is possible that MADR line differences in TAAR1 function, along with NET and SERT function, regulate stress axis response to MA in these lines of mice. MA causes the release of CRF, which then stimulates the synthesis and secretion of ACTH from the anterior pituitary into blood (reviewed in Zuloaga *et al*, 2014). Centrally, NE activity influences the CRF system through projections to the LC, amygdala, PVN, and forebrain (Makino *et al*, 2002). Peripherally, NE is produced by the adrenal medulla through sympathetic stimulation (Sedvall *et al*, 1968) and local effects of CORT (Iuvone *et al*, 1977). NE positively feeds back to the pituitary and increases the breakdown of POMCs into ACTH and β-endorphins (Itoi *et al*, 1994). ACTH release, in turn, stimulates the synthesis and release of CORT from the adrenal cortex of the kidneys (Armario, 2006; Dedovic *et al*, 2009). Preexposing MALDR mice to NET or SERT blocker prior to measuring T_1AM- or T_0AM-induced plasma CORT levels could test the relationship between NET and SERT function and TAAR1 in CORT response. If HPA response is mediated through TAAR1 regulation of monoamine transporters, CORT response may be attenuated in MALDR mice administered a transporter blocker.

MADR Line Responses to Cocaine and Ethanol

Line differences in MA intake and aversion are largely specific to MA. For example, Gubner *et al.* (2013) reported that there was no line difference in

cocaine-induced CPP or CTA. Unpublished data from the Phillips lab found that the MADR lines (replicate 1, and replicate 2 males) did not differ in two-bottle choice consumption of cocaine at 0.2, 0.4, or 0.6 g/l concentrations; however, replicate 2 female MAHDR mice consumed more cocaine than female MALDR mice at the higher concentrations (0.4, or 0.6 g/l). Additional data are needed to determine the genetic correlation between cocaine intake and MA intake in these lines. Ethanol intake was also tested in the MADR lines and found that the replicate 1 lines did not differ in consumption of a 10% ethanol solution, but that replicate 1 MAHDR mice drank significantly more 20% ethanol solution than MALDR mice in a 24-hour period. MAHDR mice of replicate 2 (selection generations 2 and 4) tended to drink more ethanol solution, but the line difference was not significant (Phillips lab; unpublished data). MALDR mice of replicate 2 (selection generation 5, relaxed generation 6) were found to have higher ethanol-induced CPP in both drug-absent and drug-present conditions (Phillips lab; unpublished data); this was not examined in replicate 1. I described in Chapter 2 that the MADR lines exhibit similar degrees of ethanol-induced hypothermia, and both lines have been found to be similarly sensitive to ethanol-induced CTA (Phillips lab; unpublished data). Thus, high and low MA drinking mice do not differ in cocaine- or ethanol-induced CTA, but there have been some differences found for consumption that have been sex-dependent or require additional study in replicate lines. These data suggest that genetic factors influencing sensitivity to the conditioned aversive effects of MA and several cocaine or ethanol traits may be somewhat distinct.

Gene Expression Differences in the MADR Lines

Genetic marker differences between the MADR lines have been examined with the intent of finding correlates with MA drinking differences, and to build QTL maps for the MA drinking phenotype (Belknap *et al*, 2013). Additionally, gene and protein expression profiles of the MADR lines have been compiled in several brain regions relevant to drug use (Belknap *et al*, 2013; Lominac *et al*, 2014; Wheeler *et al*, 2009). There are far too many genes differentially expressed between the lines to discuss or even list here (more in Table 4.2), but in relation to my dissertation and stated hypotheses, there are several genes with differential expression of particular interesting. On the chromosome 10 in the QTL region, there are 12 genes differentially expressed in the NAcc, 30 in the PFC, and 20 in the VMB. Interest and data for *Oprm1* were already described above, so I discuss others here.

Belknap *et al.* (2013) also identified differential expression of estrogen receptor 1 (*Esr1*), a gene that was reported by Wheeler *et al.* (2009) to be differentially expressed following MA. The steroid hormone signaling pathway was implicated in MA response in the MADR lines by differential expression of several genes following MA. These include upregulation of estrogen receptors (*Esr1* and *Esr2*) and the androgen receptor (*Ar*) in MAHDR, but not MALDR mice following MA (Wheeler *et al*, 2009); expression has only been measured in male MADR mice (Belknap *et al*, 2013; Wheeler *et al*, 2009). Estrogen signaling may have neuroprotective effects on MA toxicity by reducing DA efflux (Dluzen and McDermott, 2000, 2006). Sex differences in MADR line response to MA have

been described in this dissertation for CTA induced by FLUX and MA+CORT drinking (Chapter 3). One possibility is that line difference in estrogen and androgen signaling are associated with differences in these response between the sexes.

Additionally, human immunodeficiency virus type 1 enhancer binding protein 2 (*Hivep2*) was differentially expressed in MA-naïve MADR lines (Belknap *et al*, 2013). Pathway analysis of Gene Ontology identified generally downregulation of immune response pathways by MA in MALDR mice (Wheeler *et al*, 2009). Rates of HIV infection are elevated in MA-using populations, partially resulting from intravenous MA use and increased risky sexual behavior (Cheng *et al*, 2010). However, elevated rates of HIV may also exist because immune system function is reduced by direct suppression of T-cell antigen presentation, dendritic cells, and macrophages by MA (Talloczy *et al*, 2008); thus, increasing a user's risk of secondary infections, such as rapid progression of HIV. Pathway analysis of Gene Ontology also identified general downregulation of apoptotic pathways by MA in MALDR mice, and MA decreased expression of transcription factor *Nfkb2* in the NAcc, not different between lines (Wheeler *et al*, 2009). MA-naïve MAHDR mice have lower NAcc expression of *Rela*, which encodes NFκB subunit p65. NFκB transcription factor upregulation is associated with psychostimulant sensitization, which is largely similar between the lines for MA and cocaine, as discussed above (Eastwood and Phillips, 2012; Gubner *et al*, 2013; Olsen *et al*, 2013; Shabani *et al*, 2011). Additionally, mitogen-activated protein kinase kinase kinase 5 (*Map3k5*), an apoptotic signaling protein (Takeda

et al, 2008), was found to be differentially expressed in MA-naïve MADR lines (Belknap *et al*, 2013). *Mapk3*, which encodes ERK-1 MAP kinase, is expressed at lower levels in MAHDR mice following MA (Wheeler *et al*, 2009). Decreased expression of ERK-1 corresponds to increased expression of ERK-2, which is associated with drug reward and enhanced cocaine-CPP (Ferguson *et al*, 2006; Zhai *et al*, 2008), a correlation that is shared by MAHDR mice (Wheeler *et al*, 2009).

These reported differences in gene expression give insight into potential mechanisms underlying the MA drinking phenotype, but even in the case of monoamine transporter expression (as discussed in detail below), delineation of these relationships requires further investigation. A difference in gene expression between selected lines does not necessarily indicate influence on the selected trait (Belknap *et al*, 2013). Polymorphisms in a gene or a gene regulator may be responsible for the difference in expression, but not directly mediate the behavioral phenotype. An additional consideration of behavioral associations with gene expression is directionality of the relationship; changes in gene expression may result from behavior, rather than the genetic influence on behavior. For example, MA exposure increases epigenetic regulators of gene expression associated with MA-induced sensitization (Harkness *et al*, 2013), an effect that may lead to altered gene expression. MA exposure during selection could alter epigenetic markers in the germline of MADR breeder mice. This is a possibility that has not yet been explored. Although, expression measured in MA-naïve

MADR mice reduces the concern of behaviorally-driven gene expression in basal line differences reported above.

Similarly, lack of differential expression does not necessarily exclude a gene from consideration as a candidate gene for a QTL. Not included in the list of differentially expressed genes is *Taar1*, despite its presence in the Affymetrix 430 2.0 mouse GeneChips (Affymetrix) used by Belknap *et al*. (2013). As described previously, *Taar1* is within the confidence interval for the QTL on chromosome 10, and is considered to be a candidate gene for the MA drinking phenotype in MADR mice. *Taar1* would have been over-looked as a gene of interest based on expression profiling alone because although a polymorphism leads to a loss of function in the MAHDR line, the gene is not differentially expressed in the MADR lines, highlighting the necessity of multiple methods of investigation of genetic influence of complex traits.

However, the MA consumption phenotypes of the MAHDR and MALDR mice were more extreme than those of the non-selectively bred D2/B6 or transgenic mice, as indicated by the fold-difference data (Chapter 2). The influence of more genes than only *Taar1* is supported by this finding and that the chromosome 10 QTL accounts for about half, not all, of the genetically determined variance in MA intake (Belknap *et al*, 2013). For example, the *Taar1* polymorphism may interact with the expression of other genes that are or are not within the QTL interval. Inclusion of *Taar1* in network analysis of other differentially expressed genes in the MADR lines may result in gene x gene interaction that illustrate the broader involvement of *Taar1* in gene networks

commonly associated with MA-response. For example, greater expression of *Oprm1* is found in the mPFC of MALDR mice, but not in the NAcc or VMB (Belknap *et al*, 2013). As discussed above, inclusion of *Oprm1* in the network analysis reveals that its expression is regulated by top-ranking transcription factors in the MA drinking network, including *Nfkb1* (Belknap *et al*, 2013). Thus, *Oprm1* is a downstream target of other genes in the MA-intake network, which have more direct effects on MA intake, and regulate *Oprm1*, rather than *Oprm1* having direct influence on risk for MA use. While *Taar1* may have a direct influence on MA response through interactions of MA with TAAR1, it is possible that *Taar1* may be involved in a larger gene network.

Monoamine and TAAR1 Systems in Methamphetamine Intake

As detailed previously, naïve MAHDR mice have higher expression of SERT (*Slc6a4*) and NET (*Slc6a2*) in the NAcc compared to MALDR mice (Wheeler *et al*, 2009). Expression differences of some monoamine system proteins were replicated by Lominac *et al.* (2014), who reported higher expression of SERT in the NAcc core of MAHDR mice, compared to MALDR mice. This group was the first to report higher expression of DAT in the NAcc core and shell, and lower expression of D_2 receptors in the NAcc core and of 5-HT1B in the shell of MAHDR mice compared to MALDR mice. Additionally, MAHDR trended toward higher expression of 5-HT1B than MALDR mice in the mPFC. These results are important to my hypothesis that differences in monoamine transporter system function are associated with line differences in the aversive effects of MA.

MAHDR mice were found to have lower basal levels of DA in the NAcc and mPFC (Lominac *et al*, 2014). *Taar1* -/- mice also exhibit lower basal levels, in addition to greater amphetamine-induced release of DA in the striatum, compared to +/+ mice (Lindemann *et al*, 2008; Wolinsky *et al*, 2007). Acute MA increased DA in the mPFC in MAHDR mice, but not MALDR mice (Lominac *et al*, 2014). However, it is possible that basal DA-related phenotypes may not be associated with MA aversion, but rather reward sensitivity, and are therefore relevant to level of MA intake in the MADR lines.

On the other hand, differences in 5-HT disposition in MADR and *Taar1* transgenic mice do not entirely correspond. *Taar1* -/- mice have lower basal levels of 5-HT and greater amphetamine-induced 5-HT release in the dorsal striatum compared to +/+ mice (Wolinsky *et al*, 2007). MAHDR mice have higher basal levels of 5-HT in the NAcc, but show reduced sensitivity to MA-induced increases in 5-HT in the mPFC than MALDR mice (Lominac *et al*, 2014). Different brain regions and assay methods could explain discrepancies related to 5-HT. Alternatively, 5-HT may be a genetically-determined regulator of MA intake. Basal and MA-stimulated NE levels have not been measured in MADR mice. I had hypothesized that the higher expression of NET and SERT in MAHDR mice would result in greater clearance of NE and 5-HT in these mice following MA. However, a lack of difference in extraction fraction of 5-HT (and DA) between lines suggests that line differences in basal transmitter level are not related to neurotransmitter release or clearance by monoamine transporters (Lominac *et al*, 2014). Therefore, it is likely that greater transporter expression

does not lead to less transmitter in the synapse under basal conditions, which may help explain results from the NET and SERT blockade experiments in Chapter 3.

NET and SERT in Aversive Effects of Methamphetamine

The monoamine transporter genes are not located on mouse chromosome 10, and are therefore not candidates for the QTG in that region (Belknap *et al*, 2013). However, the actions of MA on NE and 5-HT systems (Bunzow *et al*, 2001; Fleckenstein *et al*, 2007; Wolinsky *et al*, 2007), implications for NET and SERT in aversive response to cocaine, (Jones *et al*, 2009; Rothman *et al*, 2001; Serafine and Riley, 2009), and implication of TAAR1 in regulation of transporter availability and disposition (Miller, 2011, 2012; Revel *et al*, 2011; Xie and Miller, 2008, 2009) made NET and SERT function viable targets for the study of MA aversion in MADR mice. Blockade of NET, and of SERT were each sufficient to induce CTA in both MADR lines. CTA developed faster in MALDR compared to MAHDR mice at the lowest dose of the SERT blocker tested. Since MAHDR were sensitive to transporter blocker-induced CTA, but not MA-induced CTA (Shabani *et al*, 2012b; Wheeler *et al*, 2009), genetic susceptibility to the conditioned aversive effects of MA vs. the transporter blockers must involve different mechanisms.

Repeated NET-blocker prior to MA-induced CTA, did not have an effect on the development of MA-induced CTA. However, repeated SERT blockade altered the development of MA-induced CTA and suggests some involvement of SERT in this phenotype. Yet, the effect of the SERT-blocker on MA-induced CTA

was considerably smaller than the effect of non-functional TAAR1, which resulted in the complete absence of MA-induced CTA (Harkness *et al*, 2015).

Jones et al (2009) found that blockers of both SERT and NET altered the development of cocaine-induced CTA. The difference between my findings with MA and those of Jones et al (2009) suggests mechanistic differences in CTA induced by MA vs. cocaine, which is consistent with our existing data for MA- and cocaine-induced CTA in the MADR lines. Based on the findings of Jones et al (2009), and because we have not seen MADR line differences in expression of the DAT encoding gene, *Slc6a3,* prior to the report by Lominac *et al.* (2014), a DAT inhibitor was not included in the current investigation because it was not expected that DAT blockade would impact MA-induced CTA. However, investigation of DAT in MA reward and aversion may be an important future direction based on the expression difference reported by Lominac *et al.* (2014), and several reports that TAAR1 regulates DAT availability and disposition (Revel *et al*, 2011; Xie and Miller, 2008) and indirectly DA signaling (Lindemann *et al*, 2008; Wolinsky *et al*, 2007). It is possible that differences in DA systems could be associated with differential sensitivity to MA reward, and not aversion.

Considerations of the Genetic Models

There are some differences in response to specific doses of MA between the replicate sets of MADR lines. For example, replicate 2 MALDR mice became more hypothermic to lower doses of MA than replicate 3 MALDR mice (Chapter 2), and plasma CORT levels in response to 2 mg/kg MA appear to be higher for replicate 2, compared to replicate 3, MALDR mice (Fig. 2.2). However, these

responses, in many cases, are qualitatively similar. These magnitude differences may reflect differences in the genetic constitution of the replicate 2 and 3 selected lines. At the time these experiments were performed, replicate 2 mice were in the 5th selected generation and maintained under relaxed selection. Replicate 2 MADR mice tested for MA-induced changes in body temperature were from relaxed selection generations 12-17, of the 5th selection generation. Although genetic drift could have led to variation in the hypothermia and HPA traits seen in the 2nd vs 3rd generation lines, line differences found in both replicates argue that these are genetic correlations reflecting phenotypic effects of MA consumption-relevant genes. However, a fourth replicate set of MADR lines is currently under development, and a future direction should be to confirm some of the findings reported here prior to significant drift.

When tested for thermal effects of MA (4 mg/kg) in a previous study, D2 mice had a hypothermic response (Grisel *et al*, 1997). Similarly, both B6 and D2 mice have been reported to become hypothermic (0.8 to 1.1°C reductions in rectal temperature) 10-20 minutes following 2 mg/kg MA, and hyperthermic at 20 mg/kg MA (Seale *et al*, 1985). Given that D2 mice were found to express a non-functional TAAR1 isoform in Chapter 2, these results seems to be in conflict with the proposed role of TAAR1 in MA-induced hypothermia. However, when several BXD strains were genotyped for *Taar1*, none were found to contain the nonsynonymous SNP (Shi *et al*, unpublished) that is found in MAHDR mice (Harkness *et al*, 2015) and in current D2 mice (Sanger, 2014). Therefore, it is unlikely that the D2 progenitor mice used to derive the BXD strains had the

mutation in *Taar1*, when those strains were developed. This suggests that the *Taar1* SNP now present in D2 mice is a mutation that arose sometime after the initial BXD RI strains were developed. The NCBI database first listed the SNP in January 2014 (dbSNP, 2015); however, selection of the first MADR replicates began in approximately 2007, and sequencing of DNA samples has confirmed the presence of the SNP in these mice. The original BXD panel was derived over 40 years ago (Gene Gene, 2015; Taylor *et al*, 1975; Womack *et al*, 1975), giving a substantial window of time when the mutation may have arisen. In fact, DNA samples from newer BXD RI mice that were provided by Dr. Rob Williams (Gene Gene, 2015), have been sequenced in the Phillips lab and confirm the existence of the *Taar1* polymorphism in some of those strains. This suggests that the polymorphism was not bred out of the older BXD strains when they were under development, but rather that it arose in the D2 strain at a later time. While this dissertation attempts to reconcile a number of traits measured in the MADR mice, *Taar1* transgenic mice, and the B6 and D2 progenitors, many more traits have been measured in the B6 and D2 mice than can be included here. As is the case with MA-induced hypothermia, it is possible that results published from D2 mice prior to the early 2000's precede the mutation of the *Taar1* SNP, and therefore, could be discrepant with more recent results.

Flanking passenger genes are a concern when introducing a transgenic region into a KO mouse, even after many generations of backcrossing (Wolfer *et al*, 2002). Identification of proximal SNPs that vary between the ES cell, blastocyst, and backcross strains can help identify or dismiss potential passenger

gene effects. The *Taar1* KO mouse strain used in this dissertation was produced with a C57BL/6N ES cell and a BALB/cJ blastocyst. Fortunately, these strains are well sequenced and behaviorally characterized, but the use of the BALB/cJ blastocyst is still less preferable than using a C57BL/6J blastocyst with the C57BL/6N ES cell. I considered the presence of polymorphisms in other genes on chromosome 10 between the two strains. For example, the *Oprm1* gene. BALB/cJ and C57BL/6J mice (similar sequence to C57BL/6N *Oprm1*) have a number of SNPs, although all polymorphisms are reported to be in intronic and untranscribed regions of the gene (Jax, 2015). Thus, the position of these SNPs reduces the concern of major changes in μ-opioid receptor function, but may still regulate receptor expression or splice variation, and is a consideration for future investigation.

Summary and Conclusions

Whether an individual develops an adverse pattern of MA use likely depends on the balance of their positive and negative experiences with the drug (Cruickshank and Dyer, 2009; Davis and Riley, 2010). I believe that the development of effective therapeutics relies on knowledge of the neural mechanisms that contribute to sensitivity to both types of motivational effects of MA. Furthermore, genetic risk factors could benefit individual prevention and treatment. This dissertation describes the first report of voluntary MA intake in animals with genetic alterations resulting in loss of TAAR1 function.

Lower genetic risk for MA consumption was associated with sensitivity to MA-induced CTA, hypothermia, and HPA response. These outcomes were

observed in two genetic models (Fig. 4.1). Transgenic mice homozygous or heterozygous for a functional *Taar1* allele avoid MA consumption, are sensitive to MA-induced CTA, hypothermia, and MA-induced plasma CORT increase. Similarly, MALDR mice, which are either homozygous or heterozygous for a functional *Taar1* allele, avoid MA consumption and are sensitive to aversive effects of MA, MA-induced hypothermia, and HPA response, Additionally, these outcomes are clear in two replicate sets of MADR lines, which strongly support common genetic influence on MA consumption and sensitivity to the aversive and hypothermic effects of MA. Furthermore, combined with data from the *Taar1* transgenic mice, these data suggest *Taar1* as a candidate gene that influences all four traits.

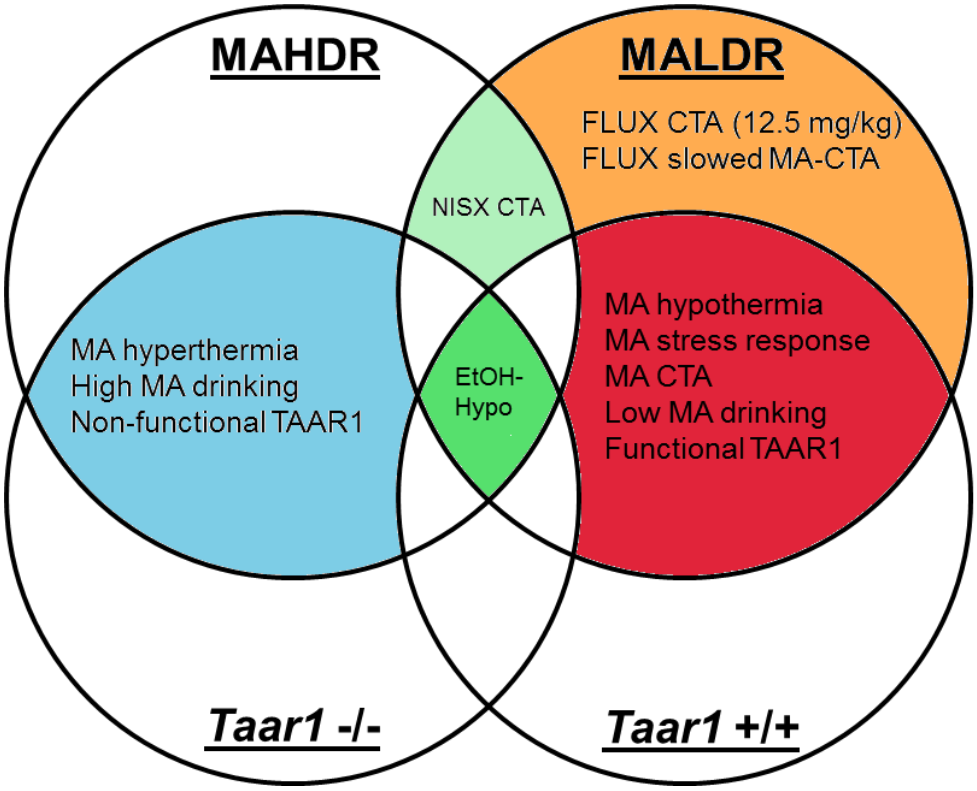

Figure 4.1. Venn diagram of MA-related traits presented in Chapters 2 and 3, organized by mouse line. Traits are shown in circles representing the genotypes found to have the trait. Traits overlapping more than one genotype are shown in the corresponding space within the associated genotypes. Traits associated with *Taar1* genotype are indicated by segregation into the left (blue region) or right (red region) pair of circles, MAHDR/*Taar1*-/- and MALDR/ *Taar1*+/+, respectively. Traits not associated with *Taar1* genotype, or differing between the MADR lines are shared by left and right circles or all four genotypes (green regions). Transporter-induced CTA was not tested in *Taar1* transgenic lines, therefore are represented in the light green and orange regions. However, I predict that *Taar1*

transgenic mice would correspond with MADR mice on these traits, in which case NISX-induced CTA would be shown in the dark green region and FLUX CTA's would be shown in the red region.

Future Directions

Aversive effects of MA, beyond MA-induced HPA and hypothermia, should be considered in future investigations. MA-induced psychosis and tachycardia may be additional aversive effects of MA associated with reduced MA-intake and may correlate with sensitivity to MA aversion in the MADR lines. MA-induced paranoid or hallucinatory states are often indistinguishable from acute paranoid schizophrenia in humans (Bell, 1973), and are experienced as distressing or aversive. Increased heart rate and blood pressure have been reported in both humans and animals exposed to MA (Harris *et al*, 2003; Hart *et al*, 2008; Hassan *et al*, 2015; Martin *et al*, 1971; Mendelson *et al*, 2006; Perez-Reyes *et al*, 1991), and is partially mediated by the actions of MA in the sympathetic nervous system (Arora *et al*, 2001; Rusyniak *et al*, 2012). While experienced as less aversive than MA-induced paranoia or hallucinations, tachycardia is distressing and can lead to health problems such as stroke (Perez *et al*, 1999), coronary heart disease, and sudden cardiac death (Kaye *et al*, 2007).

Investigation of TAAR1 in MA intake and aversion will benefit from more precise transgenic models. New techniques in transgenic production, such as CRISPR-cas9, could improve the specificity of a KO model. Combined with inducible transgenic techniques, such as Cre-lox recombination, *Taar1* genotypes could be switched on or off with an administration of Cre, and/or be isolated to specific tissues or cell types. For example, CRISPR could be used to introduce the D2 allele in reverse orientation, at the 3’ end of *Taar1* in MALDR or B6 mice, and to introduce loxP inversion sites flanking the B6 (forward) and D2

(reverse) *Taar1* genes. Thus, the administration of Cre would reverse the palindromic region and lead to expression of nonfunctional TAAR1 in mice that developed expressing functional TAAR1. Transgenic strategies such as this would improve specificity of the transgenic model and remove developmental adaptations from consideration of the behavioral findings.

Additionally, future investigations should delineate TAAR1 regulation of neurotransmitter availability, disposition, and monoamine transporter activity in the MADR mice. As described above, a DAT inhibitor was not included in the current investigation because it was not expected that DAT blockade would impact MA-induced CTA. However, more recently Lominac *et al.* (2014) reported differences in DAT expression between the MADR lines. Additionally, the role of TAAR1 in MA intake and aversion has become increasingly apparent and TAAR1 is reported to regulate availability and disposition of monoamine transporters, including DAT (Revel *et al*, 2011; Xie and Miller, 2008). It is possible that MADR line differences in DAT expression are associated with differential sensitivity to MA reward, rather than aversion.

Jing *et al*. (2015) demonstrated that a TAAR1 agonist was capable of reducing MA SA in rats. A future direction should be to investigate the ability of TAAR1 specific agonists and antagonists to manipulate MA intake and aversion in the MADR lines. However, MAHDR mice express non-functional TAAR1. Therefore, administration of a TAAR1 agonist to MAHDR mice will not have an effect on TAAR1 function. Use of specific TAAR1 antagonists to reduce MA-induced CTA, hypothermia, and CORT response in MALDR mice may provide

additional insight into the role of TAAR1 activation in the aversive response to MA. Since MALDR mice initially consume MA at the level of MAHDR mice, blocking TAAR1 from the very first exposure to MA could reduce CTA, hypothermia, and stress responses, thereby increasing their MA intake. However, TAAR1-specific antagonists are not currently commercially available and may be limited in distribution by the blood-brain barrier.

It is possible that the TAAR1 also limits MA intake in humans through increased sensitivity to aversive effects of MA. There are a number of reported synonymous and non-synonymous SNPs in the human *TAAR1* (dbSNP, 2014), but there are no reported polymorphisms that are shared across the mouse and human. Some of the reported non-synonymous SNPs in the human *TAAR1* are located in regions that should alter receptor recognition of ligand or receptor function (Pardo *et al*, 1992), but have not yet been reported to encode sub- or non-functional receptors. It is possible that the TAAR1 limits MA consumption in some humans by conferring sensitivity to aversive effects of MA. Drugs that stimulate sub-functional TAAR1 may increase aversive effects of MA and be useful for treating MA addiction. However, non-functional TAAR1 would not be expected to respond to agonists. Yet, pharmacotherapies designed to improve TAAR1 function, or TAAR1 agonists, may prove useful for reducing MA use or addiction.

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