MECHANISTIC CORRELATES OF SELECTIVE BREEDING FOR METHAMPHETAMINE INTAKE

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LIST OF ABBREVIATIONS

+/+ – wild-type genotype

+/- – heterozygous genotype

-/- – knockout genotype

5-HT – serotonin

AA – alcohol accepting selectively bred rat line

ANA – alcohol non-accepting selectively bred rat line

ANOVA – analysis of variance

ALDH2 – human alcohol dehydrogenase 2 gene

ALDH2 - alcohol dehydrogenase 2

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

B6 – C57BL/6J

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

BINA – Biphenylindanone A

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

C – Celsius

cAMP – 3'-5'-cyclic adenosine monophosphate

CC – collaborative cross

cm – centimeter

CPA – conditioned place preference

CPA – conditioned place aversion

CRISPR-Cas9 – clustered regularly interspaced short palindromic repeat (CRISPR) CRISPR-associated protein 9

CS – conditioned stimulus

CS+ – conditioned positive stimulus

CS- – conditioned negative stimulus

CTA – conditioned taste aversion

D2 – DBA/2J

D2R – dopamine D2-receptor

DA – dopamine

DAT – dopamine transporter

Dehal1 – Iodotyrosine dehalogenase 1 gene

DO – diversity outbred

DRN – dorsal raphe nucleus

EAAT2 – excitatory amino acid transporter 2

EAAT3 – excitatory amino acid transporter 3/neuronal glutamate transporter

F1 – first filial generation offspring

F2 – second filial generation offspring

FR – frequency ratio

g – gram

g/kg – grams per kilogram

GPCR – G protein-coupled receptor

h^2 – realized heritability of a quantitative trait during selective breeding

H^2 – heritability, specifically the proportion of phenotypic variance under genetic control

HDID – High drinking in the dark

HEK – human embryonic kidney cells

HEK-B6 – human embryonic kidney cells expressing the C57BL/6J trace amine-associated receptor 1

HEK-D2 – human embryonic kidney cells expressing the DBA/2J trace amine-associated receptor 1

Homer1 – homer scaffolding protein 1

Homer2 – homer scaffolding protein 2

Homer2a/b – homer scaffolding protein 2 a and b isoforms

HS – heterogeneous stock

HS/Npt – heterogeneous stock/Northport

KI – knock-in

KO – knockout

L – liter

LiCl – lithium chloride

LTD – long-term depression

LTP – long-term potentiation

μl – microliter

MA – methamphetamine

MADR – methamphetamine drinking

MAHDR – methamphetamine high drinking

MALDR – methamphetamine low drinking

Mb – megabase

MDMA – 3,4-Methylenedioxymethamphetamine

MTEP – 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine

mg – milligram

mg/kg – milligrams per kilogram

mg/L – milligrams per liter

mGluR2 – metabotropic glutamate receptor 2

mGluR5 – metabotropic glutamate receptor 5

mL – milliliter

mM – millimolar

mPFC – medial prefrontal cortex

mTAAR1-D2 – DBA/2J trace amine-associated receptor 1

mTAAR1-B6 – C57BL/6J trace amine-associated receptor 1

NAC – n-acetylcysteine

NAcc – nucleus accumbens

NAM – negative allosteric modulator

NET – norepinephrine transporter

NIH – National Institutes of Health

nM – nanomolar

Oprm1 – mouse mu-opioid receptor gene

OPRM1 – human mu-opioid receptor gene

OPRM1 – mu-opioid receptor

Oprm1^{B6} – C57BL/6J mu-opioid receptor gene allele

Oprm1^{D2} – DBA/2J mu-opioid receptor gene allele

PAM – positive allosteric modulator

PCR – polymerase chain reaction

PFC – prefrontal cortex

PKA – protein kinase A

Ppp1r14c – proteinphosphatase 1 regulatory inhibitor subunit 14c gene

Prkacb[β1]$^{-/-}$ – PKA catalytic subunit Cβ1 knockout mouse

QTG – quantitative trait gene

QTL – quantitative trait locus

R – response to selection

Rab3b – Ras-related protein Rab-3B gene

Rgs15 – regulator of G-protein signaling 17 gene

RhoA – Ras homolog family member A

RI – recombinant inbred

RNA-seq – RNA sequencing

S – cumulative selection differential

S – selection generation

SERT – serotonin transporter

shRNA – short-hairpin RNA

SNP – single nucleotide polymorphism

Taar1 – mouse trace amine-associated receptor 1 gene

Taar1^{+} – C57BL/6J trace amine-associated receptor 1 allele

Taar1^{m1J} – DBA/2J trace amine-associated receptor 1 allele

TAAR1 – trace amine-associated receptor 1

TPMT – thiopurine S-methyltransferase

TPMT – human thiopurine S-methyltransferase gene

TRPM8 – type 8 transient receptor potential ion channel

US – unconditioned stimulus

x_c^- – cystine-glutamate antiporter

VGLUT1 – vesicular glutamate transporter 1

Vip – vasoactive intestinal polypeptide gene

VIP – vasoactive intestinal polypeptide

Pcmt1 – protein-L-isoaspartate [D-aspartate] O-methyltransferase 1

VMAT2 – vesicular monoamine transporter 2

VTA – ventral tegmental area

WT – wild-type

xCT – catalytic subunit of cystine-glutamate antiporter

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Abstract

While any number of social, psychological, and economic factors contribute to methamphetamine (MA) addiction risk, numerous biological variables have also been identified. Considerable research has been dedicated to glutamate and monoaminergic signaling, specifically. The MA drinking (MADR) mouse lines were bidirectionally selectively bred for high (MAHDR) and low (MALDR) voluntary MA consumption. They were developed from an F2 cross of the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains. In addition to MA intake differences, MADR line mice differ in thermal response to MA, in their basal and MA-associated glutamate levels within the nucleus accumbens (NAcc) and medial prefrontal cortex (mPFC), and in the expression of glutamate-related proteins in the NAcc and mPFC. A quantitative trait locus (QTL) analysis identified a region on mouse chromosome 10 accounting for at least 60% of the genetic variance in MA intake between the MADR lines. Within this QTL lies the trace amine-associate receptor 1 gene (*Taar1*) and the mu-opioid receptor gene (*Oprm1*). *Oprm1* was a promising candidate for a quantitative trait gene (QTG) for MA intake differences in the MADR lines, but was later determined not to be a QTG. Rather it serves as a "hub" for regulation by the top-ranked transcription factor differential gene expression network for MA intake risk. *Taar1* was confirmed as a QTG. MAHDR line mice are homozygous for the D2 *Taar1* allele, whereas MALDR line mice are heterozygous, or homozygous for the B6 *Taar1* allele. The D2 strain contributes a *Taar1* allele, now called $Taar1^{m1J}$, with a single nucleotide polymorphism (SNP) resulting in a non-functional TAAR1. Thus, MAHDR line mice express a non-functional TAAR1, compared to the functional TAAR1 expressed by MALDR line mice.

The broad goals of this dissertation are to explore the mechanistic correlates of selective breeding for MA intake. The intent of the first aim was to further characterize the receptor encoded by $Taar1^{m1J}$. Previous work found that this receptor does not produce a cAMP response in the presence of agonist. I build on this by demonstrating the TAAR1 encoded by

Taar1^{m1J} binds ligand with drastically reduce affinity. Thus, responses to TAAR1 agonists, such as MA, in these mice are more likely due to impaired ligand binding.

The second aim focused on the hypothermic effects of addictive drugs in the MADR lines. I tested the amphetamine-like stimulant and TAAR1 agonist, 3,4-Methylenedioxy methamphetamine (MDMA), the non-amphetamine-like stimulant cocaine, and the OPRM1 agonist morphine. MDMA produced thermal responses similar to MA, characterized by hypothermia in MALDR line mice which was not observed in MAHDR line mice. Cocaine produced similar hypothermia in both MADR lines. Morphine induced hypothermia in both lines, but MAHDR line mice were significantly more sensitive to this hypothermic effect. Genotyping results from the MADR mice treated with morphine showed genetic linkage between *Oprm1* and *Taar1*, such that mice were more likely to possess *Taar1* and *Oprm1* alleles from the same progenitor strain. Data from a family of recombinant inbred mouse strains using B6 and D2 inbred strains as progenitors (BXD RI strains) supported a role for *Oprm1* genotype, but not *Taar1* genotype, in thermal response to morphine. Finally, I tested an OPRM1 partial agonist, buprenorphine, for thermal effects in the MADR lines. Buprenorphine produced hypothermia in both lines, albeit to a lesser degree than morphine. Unlike morphine, there was no clear delineation in thermal response between the lines. Genotyping results from buprenorphine-treated mice also demonstrated genetic linkage between *Oprm1* and *Taar1*. Therefore, selective breeding segregated the B6 *Taar1* allele to the MALDR line, and genetic linkage with *Oprm1* segregated the B6 *Oprm1* allele in MALDR line mice and the D2 *Oprm1* allele in MAHDR line mice. Consequently, MALDR line mice are sensitive to the hypothermic effects of TAAR1 agonists like MA and MDMA, whereas MAHDR line mice are insensitive to these effects. However, the segregation of *Oprm1* alleles has led to enhanced sensitivity to the hypothermic effects of a full agonist of OPRM1, morphine, in MAHDR line mice, but this does not translate to a partial OPRM1 agonist.

The third aim explored the role of glutamate in MA drinking of MAHDR line mice. I tested four drugs intended to facilitate proper glutamate signaling: two metabotropic glutamate receptor 5 (mGluR5) negative allosteric modulators (NAMs), a mGluR2 positive allosteric modulator (PAM), and the cystine-glutamate antiporter (x_c^-) prodrug n-acetylcysteine (NAC). All drugs failed to alter the acquisition of MA drinking. The mGluR5 NAMs were tested for their ability to attenuate established MA drinking, however they failed to do so in these experiments as well. NAC was then dissolved in drinking solutions to examine if oral NAC intake had effects on MA drinking, however oral NAC had no effect on the acquisition of MA drinking nor established MA drinking. Finally, I measured the glutamate-related proteins, mGluR5, Homer2a/b, the neuronal glutamate transporter (EAAT3), EAAT2, x_c^-, and the vesicular glutamate transporter 1 (VGLUT1) in the NAcc of MADR mice. I found no differences in the expression of any of these proteins. Thus, my data are not able to support a role of glutamate in MA intake of MAHDR line mice.

Together, these data demonstrate that, due to genetic linkage, *Oprm1* and *Taar1* alleles are often inherited from the same progenitor strain, B6 or D2. Due to selective breeding for MA intake, the *Taar1* allele from the D2 strain is sequestered in MAHDR line mice, and due to this genetic linkage the *Oprm1* allele from the D2 strain is also frequently sequestered in the MAHDR line. The B6 *Taar1* and *Oprm1* alleles are often sequestered in the MALDR line. This combination of genotypes confers resistance to MA-induced hypothermia and high MA intake in MAHDR line mice, but enhanced sensitivity to morphine-induced hypothermia and attenuated morphine intake in these mice. The *Taar1* and *Oprm1* genotypes possessed by MALDR line mice confer extreme sensitivity to MA-induced hypothermia and very low MA intake in MALDR line mice, but reduced sensitivity to morphine-induced hypothermia and greater MA intake relative to MAHDR line mice. Although glutamate plays a role in addiction, and has been implicated as a factor contributing to MA intake differences between the MADR lines, drugs intended to attenuate glutamatergic signaling failed to reduce intake in MAHDR line mice.

Chapter 1: General Introduction

History of methamphetamine

In March 2022, the Methamphetamine Response Act was signed into law, declaring methamphetamine (MA) an emergency health threat. This came after years of steadily increasing MA use and MA-related deaths. Between 2015 and 2019, MA use in the United States increased 43%, accompanied by a 180% increase in MA-related overdose deaths (Han, Compton, Jones, Einstein, & Volkow, 2021; Han, Cotto, et al., 2021). MA is an analogue of amphetamine that was first synthesized by the Japanese chemist Nagayoshi Nagai in the late 19th century, but remained fairly obscure until the 1940s (Anglin, Burke, Perrochet, Stamper, & Dawud-Noursi, 2000; Meredith, Jaffe, Ang-Lee, & Saxon, 2005; Sato, 2008). During World War II, militaries, including in the U.S., used MA to keep soldiers awake and alert (Anglin et al., 2000; Meredith et al., 2005). After the war, MA use increased drastically in Japan as surplus stock entered the market, and continued to see heavy use in Japan until the early 1950s when its distribution and use became tightly controlled (Anglin et al., 2000; Sato, 2008). In the United States in the 1960s, amphetamine-like substances (ALS) became treatments for depression and obesity, and MA was even used as a heroin addiction treatment aid (Anglin et al., 2000). Once commercial production of MA ceased, illicit drug manufacturing increased, first in the Bay Area of California (Anglin et al., 2000). As the federal government tightened restrictions and attempted to curb the production of MA, manufacturing shifted locations to elude authorities (Anglin et al., 2000). Today, The United States supply of MA comes largely from South America, and the Northwestern and Midwestern United States (Anglin et al., 2000; Meredith et al., 2005).

Sociodemographics of methamphetamine use

The striking increase in MA overdose deaths is compounded by the populations most heavily affected. Use is skewed by socioeconomic demographics. Those with lower incomes and living outside urban areas are at high risk of MA use (Han, Compton, et al., 2021; Han, Cotto, et al., 2021; Jones et al., 2020). Thus, those most likely to use MA also face the greatest

socioeconomic barriers to access treatment (i.e. money and proximity to healthcare). MA use is also highly comorbid with use of other addictive drugs (Han, Compton, et al., 2021; Han, Cotto, et al., 2021), further complicating treatment. Unsettling patterns of deaths are seen when examining them by race and ethnicity. Indigenous populations had the greatest MA-related death increase, but also started with the highest death rate (Han, Compton, et al., 2021; Han, Cotto, et al., 2021). Although MA use among black Americans is lower than among white Americans, black people experienced a higher annual increase in MA-related deaths than white people (Han, Compton, et al., 2021; Han, Cotto, et al., 2021).

The socioeconomic risk factors for MA use are correlational; to what extent MA use contributed to a specific socioeconomic position and vice versa is not known, presenting a proverbial "chicken and egg" scenario. Considering MA is also relatively inexpensive and predominantly used by populations with limited access to resources, MA may be a drug of convenience, sampled when other options are not available, and in the presence of stressful life events (Yimsaard, Maes, Verachai, & Kalayasiri, 2018). This clearly does not account for all MA use, but clinical interviews indicate the ease of obtaining MA motivates initial use and/or continued use (Baker et al., 2021; Ellis, Kasper, & Cicero, 2018; Sarani et al., 2020; Shahbazi Sighaldeh et al., 2020). This means two important things for treatment. First, addressing the socioeconomic risk factors may go a long way to curb MA use. This approach is supported by recent meta-analyses of MA treatments, which found that contingency management strategies, including paying individuals to remain abstinent, and community involvement, are some of the most effective interventions (De Crescenzo et al., 2018; Paulus & Stewart, 2020). However, application on a national scale this would require massive interventions to reduce poverty, overhaul the carceral system, and eliminate social inequalities. Addressing these kinds of structural changes are beyond the capabilities of biomedical researchers directly. Second, these socioeconomic risk factors present a barrier to treatment. By their nature they make those with the greatest risk for MA use the least likely to have access to adequate treatment, and addiction

treatment can be costly and time-consuming. Research into the biological underpinnings of MA addiction cannot remove the socioeconomic risk factors, but can work to decrease treatment costs and increase treatment efficacy.

Methamphetamine treatment

Behavioral therapies remain the most effective treatment options (De Crescenzo et al., 2018; Paulus & Stewart, 2020). Pharmacological interventions are restricted to medications to treat underlying psychological problems or ameliorate withdrawal symptoms (AshaRani et al., 2020; De Crescenzo et al., 2018; Hamel et al., 2020; Paulus & Stewart, 2020). However even with the most effective treatment options, 40-60% of participants relapse within 12 months (Brecht & Herbeck, 2014; Hser, Evans, Huang, Brecht, & Li, 2008; Lanyon, Nambiar, Higgs, Dietze, & Quinn, 2019; Paulus & Stewart, 2020).

Pharmacotherapies have proven beneficial in the treatment of addictions to some drugs. Replacement therapies (e.g. methadone) are common treatments for opioid addiction; understanding alcohol metabolism led to the use of disulfiram to treat alcohol use disorder (Pattanayak, Sagar, & Pal, 2015), and bupropion has proven beneficial as a smoking cessation aid (Wilkes, 2008). In contrast to other drugs, to date there are no federally approved pharmacological treatments for MA addiction. Modafinil is a wakefulness promoting, non-amphetamine stimulant that has been considered as a replacement therapy for MA addiction (Ballon & Feifel, 2006; Brensilver, Heinzerling, & Shoptaw, 2013; Radfar & Rawson, 2014). However, in clinical trials, modafinil has not been effective at improving MA addiction treatment outcomes (Anderson et al., 2012), though it can alleviate symptoms of MA withdrawal (McGregor, Srisurapanont, Mitchell, Wickes, & White, 2008). Methylphenidate (Ritalin™), is a dopamine (DA) reuptake inhibitor used to treat attention deficit hyperactivity disorder, and has shown some success at decreasing MA use (Brensilver et al., 2013; Tiihonen et al., 2007). Concern over abuse potential for replacement therapies limits their therapeutic potential, and raises ethical concerns when performing research in humans, since investigators may be

exposing already vulnerable populations (i.e. those with drug addictions) to addictive substances.

Drugs targeting the glutamate system have shown some promise in treating multiple drug addictions, including MA addiction. The rationale behind using these drugs is detailed later in this chapter, and in Chapter 4. To summarize, proper regulation of extracellular glutamate is required for optimal regulation of behavior (Britt et al., 2012; Kalivas, 2009; Kelley, 2004), and repeated drug use disrupts regulation of glutamate homeostasis, leading to maladaptive drug-seeking and drug-taking behaviors (Kalivas, 2009). In principle, pharmacotherapies could target mechanisms known to regulate glutamate levels and restore proper behavioral control. Among these pharmacotherapies, n-acetylcysteine (NAC) is one of the most promising (Degenhardt et al., 2016). NAC acts on the cystine-glutamate antiporter (x_c^-), from which 60% of extrasynaptic glutamate in the nucleus accumbens (NAcc) is derived (Baker, Xi, Shen, Swanson, & Kalivas, 2002). When used in conjunction with behavioral therapy, NAC reduced craving in recovering MA users (Mousavi et al., 2015), and reduced cocaine use in participants who were recently abstinent from cocaine but had started using again (LaRowe et al., 2013).

MA is a direct agonist of the trace amine-associated receptor 1 (TAAR1), which is under investigation as a treatment target for addiction and a number of other psychiatric disorders (Schwartz et al., 2018). TAAR1 will be discussed in greater detail later in this chapter, but its clinical value derives from its role in regulation of monoaminergic transmission, particularly DA (Bradaia et al., 2009; Revel et al., 2011; Schwartz et al., 2018; Xie & Miller, 2008; Xie & Miller, 2009a, 2009b). Drugs targeting TAAR1 as a treatment for addiction have not been tested in humans, but preclinical studies are promising. In one study, a TAAR1 agonist decreased psychomotor-sensitization to MA in rats, and reduced MA self-administration (Jing, Zhang, & Li, 2015). When rats were treated with a TAAR1 partial agonist, which occupies and activates the receptor with partial efficacy (48%–73% maximum) (Revel, Moreau, et al., 2012), they would not work as hard for MA, and the partial agonist reduced reinstatement of MA seeking (Pei, Asif-

Malik, & Canales, 2016). Human TAAR1 polymorphisms that differ in their level of response to TAAR1 agonists have been identified (Shi et al., 2016), and a common *TAAR1* variant was associated with increased MA craving in both actively using MA addicted participants and those in remission (Loftis et al., 2019). Aside from addiction, TAAR1 has garnered attention as a treatment target for the positive symptoms of schizophrenia (Revel et al., 2012, 2013; Schwartz et al., 2018), obsessive compulsive disorder (Schwartz et al., 2018; Sukhanov et al., 2019), and depression (Revel et al., 2013; Schwartz et al., 2018). This offers a possibility of simultaneously treating addiction and comorbid disorders.

In addition to pharmacotherapies, understanding the biology of MA addiction may aid in identifying biological risk factors and to customize treatment. We have seen such risk identification in alcohol addiction research. Those lacking alcohol dehydrogenase 2 (ALDH2) are significantly less likely to abuse alcohol (Choi et al., 2006; Crabb, Edenberg, Bosron, & Li, 1989; Edenberg & McClintick, 2018; Mayfield, Harris, & Schuckit, 2009). Simple genetic tests can identify these individuals by which variant of *ALDH2* they possess. While this is an example of biological protection against an addiction, in principle, biological risks can be identified should they exist. The aforementioned human *TAAR1* variants, with a common variant associated with increased MA craving, is an example (Loftis et al., 2019). Greater understanding of biological risk and protection can also inform the treatment approach. Just as psychological factors may vary between patients, so might biological factors. Accounting for both psychological and biological variables when developing treatment plans may improve outcomes.

Immunotherapies are also being investigated. In a way, this is a more direct and simpler approach to treating addiction. Such therapies have the potential to treat overdose, treat relapse, and inoculate at-risk individuals (Harwood & Myers, 2004; Hossain, Hassanzadeganroudsari, Kypreos, Feehan, & Apostolopoulos, 2021; Kosten & Owens, 2005; Xu & Kosten, 2021; Zalewska-Kaszubska, 2015). These therapies exploit the body's natural immune system (active immunization) to develop antibodies to a specific drug, the same way

vaccines do, or they can use monoclonal antibodies (passive immunization), which still bind to the target drug but have a shorter duration of action (Harwood & Myers, 2004; Kosten & Owens, 2005). These therapies carry no abuse potential, and should require less effort for patients, as they would only have to get the initial vaccine and occasional boosters if active vaccination is used. Immunotherapies showed promise early on, but there remain few clinical trials, and trials infrequently demonstrate treatment potential (Hossain et al., 2021; Xu & Kosten, 2021; Zalewska-Kaszubska, 2015). Monoclonal antibody-based vaccinations can be helpful in treating overdose, but have shown limited efficacy as a long-term treatment option (Hossain et al., 2021; Xu & Kosten, 2021). In humans, it is difficult to achieve effective antibody titer levels with active vaccination (Hu, Zheng, Huang, & Zhang, 2014; Martell et al., 2009; Xu & Kosten, 2021; Zalewska-Kaszubska, 2015). Furthermore, a patient can increase the dose of the target drug, saturating available antibodies, to achieve the desired effects. Even with low antibody titer levels, active vaccines may help prevent unintentional overdose and mitigate toxic effects of a drug (Hossain et al., 2021; Xu & Kosten, 2021). No MA vaccines have been tested in humans, but some active vaccines show promise in rodents (Hossain et al., 2021).

Subjective and physiological effects of MA

Amphetamine and low to moderate doses of MA induce alertness, stimulation, reduce fatigue, cognitive improvement, disinhibition, relaxation, euphoria, and reduce hunger (Cruickshank & Dyer, 2009; Hart et al., 2008; Martin, Sloan, Sapira, & Jasinski, 1971; May, Aupperle, & Stewart, 2020; Nordahl, Salo, & Leamon, 2003). However, anxiety and paranoia are also experienced, particularly at higher doses (Bell, 1973; Cruickshank & Dyer, 2009; Martin et al., 1971; Zweben et al., 2004), as are hallucinations and delusions (Bell, 1973; Cruickshank & Dyer, 2009; Zweben et al., 2004).

In humans, many of the physiological effects of MA mirror typical sympathetic nervous system activation, and are characterized by increased heart rate, blood pressure, respiration, and pupil dilation (Cruickshank & Dyer, 2009; Hart et al., 2008; Martin et al., 1971), and can

produce hyperthermia (Cruickshank & Dyer, 2009; Martin et al., 1971). Physiological effects intensify with dose, resulting in tachycardia, hyperventilation, and heart palpitations (Cruickshank & Dyer, 2009; Gray, Fatovich, McCoubrie, & Daly, 2007; Hart et al., 2008; Martin et al., 1971).

The subjective and physiological effects motivating use vary among individuals. Stimulation and perceived cognitive-enhancing effects of amphetamine among college students are reported as reasons to use amphetamines (Haas, Momo, Dias, Ayodele, & Schwarzbold, 2019; McCabe, Knight, Teter, & Wechsler, 2005). Stimulation and euphoria motivate recreational MA use (Díaz, Heckert, & Sánchez, 2005; Ellis et al., 2018; Shahbazi Sighaldeh et al., 2020). For many, MA has a utilitarian use. Some initiate MA use to stay awake and increase productivity (Shahbazi Sighaldeh et al., 2020), or enhance sexual performance (Baker et al., 2021; Díaz et al., 2005; Shahbazi Sighaldeh et al., 2020). MA is increasingly being used by those with opioid addictions (Baker et al., 2021; Ellis et al., 2018), often to enhance the “high” of opioids or balance the depressive effects of opioids, treat withdrawal symptoms, or as a cheaper alternative to opioids (Baker et al., 2021; Ellis et al., 2018). Many MA users report symptoms of anxiety and depression, that may or may not be related to their MA use, but regardless use MA to alleviate those symptoms (Darke, Kaye, McKetin, & Duflou, 2008; May et al., 2020; Zweben et al., 2004). Anxiety and depressive symptoms increase with repeated MA use, promoting further use to alleviate those symptoms (Darke et al., 2008).

Patterns and consequences of MA use

Most people who use methamphetamine use multiple times a week (McKetin, Kelly, & McLaren, 2006; Simon et al., 2002). Generally, people use MA 1-4 times per day, starting in the morning (McKetin et al., 2006; Simon et al., 2002). Use can exceed 10 times per day though (Simon et al., 2002). The preferred route of administration varies by study. Inhalation and injection are the most common routes of administration, but intranasal and oral administration are often preferred (McKetin et al., 2006; Simon et al., 2002).

Enhanced glutamate accumulation resulting from MA induces excitotoxicity (Moratalla et al., 2017; Yamamoto, Moszczynska, & Gudelsky, 2010). MA also generates reactive oxygen species and reactive nitrogen species which can damage cellular organelles (Lin, Kang, Wong, Mao, & Wan, 1999; Northrop & Yamamoto, 2015; Obata, 2002; Yamamoto et al., 2010; Yang et al., 2018). MA increases blood-brain barrier permeability allowing other neurotoxic insults (Northrop & Yamamoto, 2015). MA use causes liver damage, increasing circulating ammonia, which is known to enhance MA neurotoxicity (Halpin, Northrop, & Yamamoto, 2014; Halpin & Yamamoto, 2012; Northrop & Yamamoto, 2015). Damage to the cardiovascular system is a hallmark of MA use. MA increases blood pressure (Cruickshank & Dyer, 2009; Martin et al., 1971; Shappell, Kearns, Valentine, Neri, & DeJohn, 1996), and high doses can lead to myocardial infarction (Cruickshank & Dyer, 2009; Waksman et al., 2001). Repeated MA use greatly increases the chance of heart disease (Karch, Stephens, & Ho, 1999; Kevil et al., 2019), leaving regular MA users with a 20% - 30% increased risk of stroke and heart attack (Kevil et al., 2019; Parekh et al., 2018).

Individuals with a history of MA use also show cognitive impairments. Recently abstinent MA users exhibit poorer performance on simple motor tasks and in auditory verbal learning tasks than control participants (Volkow, Chang, Wang, Fowler, Leonido-Yee, et al., 2001), and display memory deficits (Nordahl et al., 2003; Simon et al., 2000). A recent review of cognitive effects of MA in animals found consistent deficiencies in working memory (Braren, Drapala, Tulloch, & Serrano, 2014; Mizoguchi & Yamada, 2019; Nagai et al., 2007) and recognition memory (Kamei et al., 2006; Long et al., 2017; Mizoguchi & Yamada, 2019), and found that MA exposure led to riskier decision making (Mizoguchi, Wang, Kusaba, Fukumoto, & Yamada, 2019; Mizoguchi & Yamada, 2019). Chronic MA use can lead to psychiatric symptoms as well. A study of abstinent MA users found many had experienced psychosis during MA use, and some experienced persistent symptoms such as hallucinations and delusions (Sekine et al., 2006). In humans, a history of MA use is associated with maladaptive behaviors including

aggression, uncooperativeness, and disorganization, the severity of which increases with a longer history of MA use (Sekine et al., 2001, 2006). Studies in humans and animals repeatedly demonstrate chronic MA use depletes serotonin (5-HT) and dopamine (DA) (Hotchkiss & Gibb, 1980; Meredith et al., 2005; Northrop & Yamamoto, 2015; Ricaurte, Schuster, & Seiden, 1980), and decreases 5-HT (SERT) and DA (DAT) transporter levels (Northrop & Yamamoto, 2015; Sekine et al., 2001, 2006; Volkow, Chang, Wang, Fowler, Franceschi, et al., 2001; Volkow, Chang, Wang, Fowler, Leonido-Yee, et al., 2001).

Pharmacokinetics and pharmacodynamics

MA is more lipophilic than amphetamine, enhancing brain penetration (Barr et al., 2006). In humans, within 10-20 minutes of injection, plasma and brain concentrations of MA reach their peak, but have a slow rate of clearance (Cruickshank & Dyer, 2009; Newton, De La Garza, Fong, et al., 2005; Shappell et al., 1996; Volkow et al., 2010). Peak cardiovascular and subjective effects can occur within 15 minutes of injected or intranasal MA administration, often preceding peak plasma concentrations, but it can take several hours before peak cardiovascular and subjective effects are experienced after oral MA administration (Cruickshank & Dyer, 2009; Newton, De La Garza, Kalechstein, & Nestor, 2005; Shappell et al., 1996). Bioavailability in humans is highly variable (estimates between 37% and 90%) (Cook et al., 1993, 1992; Harris et al., 2003), and depends not just on the route of administration (e.g. intravenous vs inhaled) but also the dose and exact method of administration (e.g. pipe size and temperature of inhaled MA) (Cook et al., 1993, 1992; Harris et al., 2003). The elimination half-life of smoked, injected, or intranasal MA is 10-12 hours (Cook et al., 1993, 1992; Harris et al., 2003; Schepers et al., 2003; Shappell et al., 1996). MA is predominantly metabolized in the liver by cytochrome P4502D6, and within 24 hours the majority of the initial dose is excreted in urine (Caldwell, 1976; Cruickshank & Dyer, 2009; Lin et al., 1997; Schep, Slaughter, & Beasley, 2010). Rodents metabolize MA significantly faster than humans. In mice, peak MA plasma levels occur 15 minutes after an intraperitoneal injection, and MA is no longer detectible by 4 hours (Shabani,

McKinnon, Cunningham, & Phillips, 2012). The plasma half-life of intravenous MA is approximately 70 minutes in rats (Cho, Melega, Kuczenski, & Segal, 2001), and 35 minutes in mice (Wagner, Shireman, Ahn, Shen, & Wang, 2018).

Monoamine transporters

MA acts as a substrate for monoamine transporters, competing for transport with endogenous monoamines. It has greatest affinity for the norepinephrine transporter (NET), followed by the DAT, and has lowest affinity for SERT (Rothman et al., 2001; Sulzer, Sonders, Poulsen, & Galli, 2005). Competition at these transporters prevents reuptake of monoamines, resulting in their accumulation in the synapse, and MA is transported into the cell in their stead (Kahlig et al., 2005; Sitte, Reither, Singer, & Pifi, 1998). MA can have additional effects on monoamine transporters via TAAR1, which will be explained shortly.

Vesicular monoamine transporters

Once inside the cell, MA disrupts the vesicular monoamine transporter 2 (VMAT2), causing the release of monoamines into the cytosol and subsequent release into the synapse (Rothman et al., 2001; Sulzer et al., 2005). The exact mechanisms by which MA achieves this have not been fully elucidated. One hypothesis, the weak base hypothesis, posits that MA disrupts the proton gradient between the cytosol and internal vesicle environment (Panenka et al., 2013). Proper VMAT2 function requires a slightly acidic evironment (Panenka et al., 2013). MA accumulates in the vesicle and, as a weak base, alters the pH thereby disrupting monoamine sequestration (Panenka et al., 2013). However, concentrations of MA would have to be so high to disrupt VMAT2 (Panenka et al., 2013; Schwartz, Weizman, & Rehavi, 2006) that this hypothesis cannot fully explain MA-induced monoamine release. MA also competes with endogenous monoamines at VMAT2, thereby preventing uptake into the vesicle and permiting them to be reverse tranported out of the cell (Panenka et al., 2013; Schwartz, Weizman, & Rehavi, 2006).

Trace amine-associated receptor 1

MA is an agonist for TAAR1 (Bunzow et al., 2001; Wolinsky et al., 2007). TAAR1 is an intracellular G protein-coupled receptor (GPCR), thus activation requires a ligand present within the cell. Among addictive drugs, the ability to enter the cell and activate TAAR1 appears to be unique to amphetamine-like substances, including MA (Harkness, Shi, Janowsky, & Phillips, 2015). TAAR1 is recognized as a regulator of monoaminergic activity (Bradaia et al., 2009; Revel et al., 2011; Xie & Miller, 2008; Xie & Miller, 2009b). TAAR1 activation reduces monoamine transporter function inhibiting monoamine uptake, and induces monoamine efflux in cell culture and striatal synaptosomes of monkeys and mice (Underhill, Colt, & Amara, 2020; Xie & Miller, 2008).

TAAR1 exists in two intracellular compartments, one that couples to $G\alpha_s$ subunits and one to $G\alpha_{13}$ subunits (Underhill et al., 2019). Activation of $G\alpha_{13}$-coupled TAAR1 activates the small GTPase Ras homolog family member A (RhoA), which then internalizes DAT, the norepinephrine transporter (NET), and the neuronal glutamate transporter (EAAT3) (Underhill et al., 2019, 2020; Wheeler et al., 2015). It was recently reported that TAAR1 activation internalizes SERT as well (Underhill & Amara, 2022). Whole-cell patch-clamp recordings of substantia nigra DA neurons indicate that amphetamine-induced EAAT3 internalization potentiates NMDA- and AMPA-dependent evoked glutamatergic currents (Underhill et al., 2014). How this relates to overall neuronal activity has not been thoroughly investigated. Potentiation at these synapses would not necessarily increase DA neuron firing. Instead, it may alter the firing pattern when there is a glutamatergic signal, and/or make the DA neuron more susceptible to glutamatergic inputs. $G\alpha_s$-coupled TAAR1 activation stimulates cyclic AMP (cAMP) production, which in turn facilitates the phosphorylation of RhoA by protein kinase A (PKA), halting RhoA-mediated transporter internalization (Underhill et al., 2020, 2019). The $G\alpha_s$-mediated signaling and $G\alpha_{13}$-mediated signaling act in parallel (Underhill et al., 2019), but there is a delay between the two. $G\alpha_{13}$ signaling creates an initial increase in RhoA activation, and subsequent decrease in surface DAT, NET, SERT, and EAAT3. After about 30 minutes, the

effects of $G\alpha_s$ signaling manifest in increased RhoA phosphorylation and a decrease in activated RhoA (Underhill et al., 2019; Wheeler et al., 2015). A protein kinase C (PKC)-mediated pathway is also engaged, but whether or not this is related to $G\alpha_{13}$-coupled or $G\alpha_s$-coupled TAAR1 signaling is not known (Xie & Miller, 2007). The PKC pathway reverses DAT transport, causing an efflux of DA into the extracellular space (Xie & Miller, 2007). PKC and RhoA activation can be independent, or PKC can facilitate RhoA complex formation with downstream effectors (Barandier, Ming, Rusconi, & Yang, 2003). TAAR1-dependent PKC effects may thus be independent of RhoA activation, or could be part of the same signaling cascade. $G\alpha_{13}$-coupled TAAR1 activation could thus recruit both PKC and RhoA, leading to both monoamine transporter internalization and reverse transport. If this is the case, the $G\alpha_s$-mediated TAAR1 signaling pathway may also terminate DA efflux caused by reversal of DAT (Xie & Miller, 2007).

Somewhat paradoxically, TAAR1 activation is also known to decrease activity of monoamine neurons (Espinoza et al., 2011; Leo et al., 2014; Lindemann et al., 2005), and extracellular in vivo measures in mice striatum find that TAAR1 actually dampens amphetamine-induced monoamine release (Lindemann et al., 2008). The exact mechanism behind this is not known. It may be due to TAAR1 inhibiting excitatory projections to TAAR1-containing neurons (Revel, Meyer, et al., 2012). Autoreceptor activation is another possibility. The research in this area is almost exclusively in DA neurons, but all the autoreceptor-mediated effects that will be discussed could apply to any monoamine autoreceptor. There is ample evidence implicating an interaction between DA D2-receptor (D2R) autoreceptors and TAAR1 (Espinoza et al., 2011; Geracitano, Federici, Prisco, Bernardi, & Mercuri, 2004; Harmeier et al., 2015; Leo et al., 2014; Xie, Westmoreland, & Miller, 2008). An increase in extracellular DA due to TAAR1-mediated changes in DAT function may not be sufficient to activate post-synaptic receptors, but could be sufficient to activate D2R autoreceptors, inhibiting further DA release into the synapse and ultimately reducing DA signaling. The data support this. D2R agonists inhibited TAAR1 activity

in cultured cells and mouse brain slices (Leo et al., 2014; Xie et al., 2008), and a D2R antagonist increased TAAR1 signaling in cultured cells (Espinoza et al., 2011). An earlier study did not look at TAAR1 activity intentionally, but rather the effects of trace amines on rat midbrain DA neurons (Geracitano et al., 2004). Trace amines decreased DA neuron activity, and this reduction was blocked by a D2R antagonist (Geracitano et al., 2004). Direct interactions between D2R and TAAR1 have been a focus of several studies (Espinoza et al., 2011; Harmeier et al., 2015). Purportedly, a TAAR1-D2R heterodimer can form (Harmeier et al., 2015). This TAAR1-D2 heterodimer reportedly enhances D2R agonist affinity and potency (Harmeier et al., 2015). A similar TAAR1-driven increase in affinity and potency of agonists for 5-HT autoreceptors has also been observed, albeit dimerization with TAAR1 was never reported (Espinoza et al., 2011). However, a TAAR1-D2R heterodimer has only been observed once using procedure capable of detecting physical interactions between proteins (e.g. western blotting) (Harmeier et al., 2015). D2Rs are also localized to the plasma membrane, but TAAR1 is well-known to be intracellular (Bunzow et al., 2001; Miller, 2005; Xie & Miller, 2009b). A physical interaction between TAAR1 and plasmalemmal receptors would require trafficking mechanisms yet to be observed. So, while evidence that D2R autoreceptors, and perhaps all monoaminergic autoreceptors, can functionally “throttle” TAAR1 activity, there is not consistent evidence for a physical interaction between TAAR1 and autoreceptors.

While the literature for TAAR1 effects on monoaminergic neurons often focuses on DA, 5-HT, and norepinephrine separately, the evidence is consistent that TAAR1 activation reduces monoamine transporter function, and can reverse transporter function (Xie & Miller, 2007). DAT, NET, and SERT internalization have already been confirmed (Underhill & Amara, 2022; Underhill et al., 2020, 2019). TAAR1 reverses DAT, but NET and SERT reversal have not be examined. However, considering all the evidence to date suggests TAAR1 has no specificity for a particular monoamine transporter, it is likely that TAAR1 activation would reverse all monoamine transport. There is also a clear role for monoamine autoreceptors in the dampening

effect TAAR1 activation has on monoamine neurons. From all this, a general model for TAAR1 modulation of monoamine neurons can be formed (Figure 1.1). In this model, a TAAR1 agonist activates TAAR1 in a monoamine neuron. This agonist could be any trace amine, a monoamine, or an exogenous agonist like MA. Activating TAAR1 leads to recruitment of PKC/RhoA and internalization of plasmalemmal transporters, and reversal of remaining monoamine transporters. This increases extracellular monoamines and glutamate. At the same time, the increase in extracellular monoamines activates autoreceptors, inhibiting monoamine release and inhibiting TAAR1 signaling. TAAR1 activation also engages a PKA-mediated signaling pathway, which halts the PKC/RhoA pathway and consequently inhibits transporter internalization and reversal. This PKA-mediated pathway is engaged independent of the PKC/RhoA pathway, however there is a delay between the effects of the PKC/RhoA pathway and those of the PKA pathway. This leads to a short increase in extrasynaptic monoamines, but an overall decrease in monoaminergic signaling. While this is happening, EAAT3 internalization potentiates glutamatergic signaling onto monoamine neurons, should a glutamate afferent exist. The origin of the glutamatergic afferent depends on where the monoamine neuron is located. For instance, numerous brain regions send glutamatergic projections to ventral tegmental area (VTA) DA neurons projecting to the PFC and NAcc (Britt et al., 2012; Kalivas, 2009; Morales & Margolis, 2017). These specific connections mediate reward and aversion, (Morales & Margolis, 2017), so potentiation of the glutamatergic signaling at these synapses would modulate any subsequent DA signaling from the VTA that is important for reward/aversion processing.

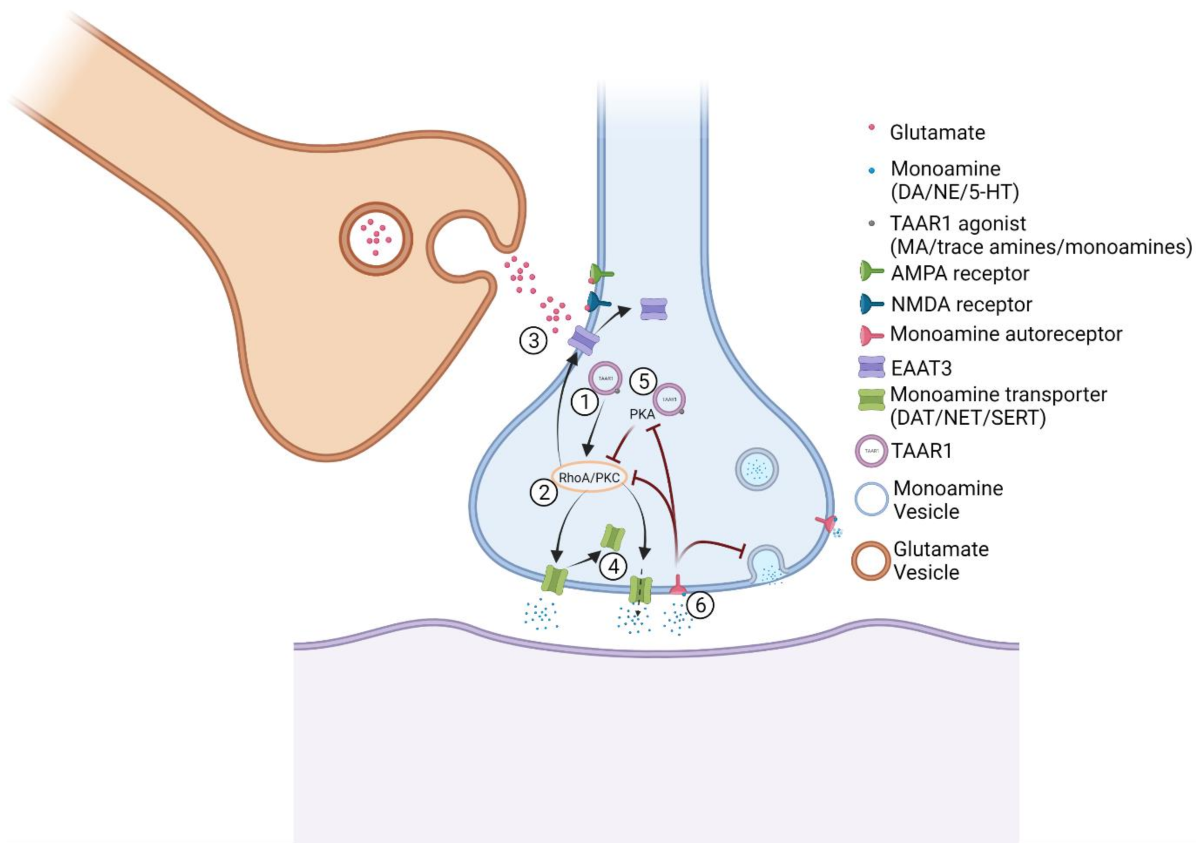

Figure 1.1. Proposed TAAR1 signaling mechanisms and effects on monoamine neurons. (1) TAAR1 is tonically activated by trace amines, but can be activated by monoamines, or by exogenous agonists including amphetamine and amphetamine like substances, such as MA. TAAR1 agonists activate TAAR1 in 2 intracellular pools. (2) One intracellular pool leads to the activation of RhoA and engagement of PKC, in which case, (3) EAAT3 is internalized. EAAT3 internalization requires PKC, but involvement of RhoA has not been verified. EAAT3 internalization increases extracellular glutamate originating from glutamatergic projections from yet unidentified brain regions. This increases AMPA and NMDA receptor activation. Glutamate projections to monoamine neurons are part of circuits important for reward and aversion processing. Furthermore, MA increases glutamate in several brain regions with large monoaminergic neuron populations. (4) RhoA activation also internalizes monoamine transporters, and PKC signaling reverses monoamine transport causing monoamine efflux. Both

increase extracellular monoamines. (5) The other intracellular TAAR1 pool recruits PKA, halting the RhoA/PKC signaling cascade and consequently halting EAAT3 and monoamine transporter internalization and monoamine efflux. This happens in parallel with the RhoA/PKC signaling cascade but the effect is delayed relative to RhoA/PKC effects on transporters, leaving a window during which transporter activity is disrupted. (6) Extracellular monoamines, increased due to transporter internalization and monoamine efflux, can activate monoamine autoreceptors. Autoreceptor activation inhibits both release of monoamines from vesicles and TAAR1-dependent signaling. This results in a net reduction in monoaminergic activity. MA: methamphetamine; TAAR1: trace amine-associated receptor 1; AMPA: Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid; NMDA: N-methyl-D-aspartate; EAAT3: neuronal excitatory amino acid transporter 3; DA: dopamine; NE: norepinephrine; 5-HT: serotonin; DAT: dopamine transporter; NET: norepinephrine transporter; SERT: serotonin transporter; RhoA: Ras homolog family member A; PKA: cAMP-dependent protein kinase; PKC: protein kinase C.

Neurocircuitry of MA use

MA's effects on DA transmission perturbs signaling in the mesocorticolimbic pathway, circuitry vital for reward processing (Kalivas, 2009; Völlm et al., 2004). This includes DA projections from the VTA to the NAcc (Feltenstein & See, 2008; Kalivas, 2009; Le Moal & Koob, 2007; Wise, 2004, 2009). The DA projections from the VTA to the NAcc signal reward, and the aforementioned actions of MA on DA neurons elevate NAcc DA (Feltenstein & See, 2008; Kalivas, 2009; Le Moal & Koob, 2007; Wise, 2004, 2009).

DA transmission is required for the rewarding effects of addictive drugs, but glutamate transmission is necessary for learning and goal directed behaviors, including those associated with drug-seeking (Kalivas, 2007, 2009; Kalivas & Volkow, 2005). Glutamate is the most abundant excitatory neurotransmitter in the brain, and is involved in learning and memory formation. Kalivas et al. (2009) proposes a glutamate homeostasis hypothesis of addiction, wherein repeated drug administration dysregulates glutamate signaling necessary for adjusting behaviors for changing contingencies (Kalivas, 2009). Since the glutamate homeostasis hypothesis was proposed, numerous studies have confirmed dysregulation of glutamate resulting from chronic drug exposure in both humans and animals. These changes are broad, and involve alterations in the pre- and post-synaptic terminals, glutamate synthesis and release, and glutamate metabolism (Chen et al., 2021; Chiamulera, Piva, & Abraham, 2021; Fischer, Knackstedt, & Rosenberg, 2021)

Amphetamine stimulates glutamate release in the NAcc and VTA (Giorgetti, Hotsenpiller, Ward, Teppen, & Wolf, 2001; Reid, Hsu, & Berger, 1997). Along with the prefrontal cortex (PFC) and amygdala, these regions comprise a limbic subcircuit of the corticostriatal circuit (Everitt & Robbins, 2005; Kalivas, 2009; Koob & Volkow, 2016). In addition to the DA projections sent from the VTA, this limbic subcircuit involves glutamatergic projections from the amygdala and PFC to the NAcc, and reciprocal glutamatergic connections between the amygdala and PFC (Everitt & Robbins, 2005; Kalivas, 2007, 2009; Kalivas & Volkow, 2005). The NAcc serves as a

“gateway” between the limbic circuit and motor outputs, and the rest of the limbic circuit exerts control over behaviors via glutamatergic projections to the NAcc (Everitt & Robbins, 2005; Kalivas, 2007, 2009; Kalivas & Volkow, 2005). Changes in glutamate signaling and related proteins due to addictive drug exposure, including MA, are broad and encompass nearly every aspect of glutamate transmission and regulation (Chen et al., 2021; Chiamulera et al., 2021; Fischer et al., 2021; Kalivas, 2009; Márquez et al., 2017; Murray, Everitt, & Belin, 2012). Perturbations of glutamate signaling are more thoroughly detailed in Chapter 4, but some general changes are worth noting here. Repeated drug exposure leads to decreased long-term potentiation (LTP) (Bowers et al., 2004; Kalivas, 2009; Moussawi et al., 2009; Xi, Baker, Shen, Carson, & Kalivas, 2002) and long-term depression (LTD) in NAcc synapses of rodents (Kalivas, 2009; Kasanetz et al., 2010; Lüscher & Huber, 2010; Martin, Chen, Hopf, Bowers, & Bonci, 2006). The decrease in LTP arises from persistent potentiation of these synapses, which occludes LTP (Bowers et al., 2004; Moussawi et al., 2009; Xi et al., 2002). The attenuation of LTD is likely compensatory, as pharmacologically decreasing LTD inhibits self-administration and seeking of addictive drugs, including MA (Brown, Stagnitti, Duncan, & Lawrence, 2012; Gass, Osborne, Watson, Brown, & Olive, 2009; Herrold, Voigt, & Napier, 2013; Niedzielska-Andres et al., 2021; Osborne & Olive, 2008; Palmatier, Liu, Donny, Caggiula, & Sved, 2008). Kalivas (2009) details various molecular changes to the glutamate system, but a few stand out for their role in LTP and LTD disruptions and their promise as targets for pharmacotherapies (Kalivas, 2009). In a drug-naïve state, x_c^- provides the majority of the extrasynaptic glutamate in the NAcc (Baker et al., 2002). The glutamate derived from x_c^- typically activates the metabotropic glutamate receptor 2 (mGluR2) and the metabotropic glutamate receptor 5 (mGluR5) (Kalivas, 2009; Kupchik et al., 2012; Moran, McFarland, Melendez, Kalivas, & Seamans, 2005; Moussawi et al., 2009; Xi et al., 2002). mGluR2 is a source of LTP and functions as an autoreceptor, inhibiting glutamate release (Grover & Yan, 1999; Kalivas, 2009; Wu, Rowan, & Anwyl, 2004), and mGluR5 activation generates LTD (Conn & Pin, 1997; Kalivas,

2009; Malenka & Bear, 2004; Nicoletti et al., 2011). After repeated drug exposure, membrane-bound x_c^- is reduced in the NAcc, thereby substantially decreasing extrasynaptic glutamate (Kalivas, 2009; Kau et al., 2008; Moussawi et al., 2009; Xi et al., 2002), reducing glutamatergic tone on mGluR2 and mGluR5. Consequently, the typical LTD provided by mGluR5 is reduced as is the attenuation of glutamate release from mGluR2 activation (Kalivas, 2009; Kasanetz et al., 2010; Lüscher & Huber, 2010; Moran et al., 2005; Xi et al., 2002). This is exacerbated by reductions in membrane-bound mGluR2 and mGluR5 (Kalivas, 2009; Kasanetz et al., 2010; Lüscher & Huber, 2010; Moran et al., 2005; Xi et al., 2002). This provides the rationale behind clinical applications of NAC. NAC is a prodrug for x_c^-, and helps to restore some of the lost extrasynaptic glutamate (Kalivas, 2009; Knackstedt et al., 2009; McClure, Gipson, Malcolm, Kalivas, & Gray, 2014; Moran et al., 2005). This activates the remaining membrane-bound mGluR2 and mGluR5, thus helping regain some of the typical mediation of glutamate signaling.

The glutamatergic projections from the PFC to the NAcc are particularly important for modifying behaviors in the presence of changing contingencies (Kalivas, 2009). The disruptions in glutamate signaling therefore prevent the proper modification of behavior when, for instance, seeking and taking a drug becomes detrimental. This has motivated research into pharmacotherapies, such as NAC, intended to regain glutamate homeostasis and hopefully assist patients in regaining control of drug-motivated behaviors (McKetin et al., 2017).

Behaviors and animal models for studying addiction

Operant drug self-administration

Operant self-administration is the "gold standard" for assessing the reward value of a drug (Sanchis-Segura & Spanagel, 2006). Operant self-administration is rooted in the principles of operant conditioning. This originated with Edward Thorndike in the early 20th century, but the study and characterization of reinforcement schedules was developed by B.F. Skinner in the 1930s (Sanchis-Segura & Spanagel, 2006). In its simplest form, operant self-administration consists of a single device (manipulandum) an animal must manipulate to trigger reinforcer

delivery, such as a lever or nose-poke (Sanchis-Segura & Spanagel, 2006). In drug self-administration the reinforcer is, clearly, the drug of interest. Various methods can be used to deliver the drug, and may depend on the experimental question.

Operant self-administration permits different aspects of drug reinforcement to be probed, based on the schedule of reinforcement (Sanchis-Segura & Spanagel, 2006). Frequency ratio (FR) 1 schedules are easy to train and simply deliver one unit of the drug per response on the manipulandum. The extents an animal will go to obtain a drug can be measured by increasing the response effort, such as increasing the number of times a lever needs to be pressed to get the reward. Any number of additional manipulanda and additional features can be added to better address experimental needs. Two or more manipulanda could be added to dissociate any reinforcing effects of the manipulanda themselves and the drug (Sanchis-Segura & Spanagel, 2006), or cues (e.g. lights or sounds) can be presented to address questions of associative learning (Sanchis-Segura & Spanagel, 2006). Operant self-administration is limited by the method of drug delivery, since delivery methods like intravenous delivery require surgery, which can be challenging, particularly in the mouse. It also requires specialized equipment that can be expensive and unavailable to a particular lab.

Two-bottle choice drinking

Two-bottle choice drinking allows animals to voluntarily consume a drug, permitting measures of total drug intake but also drug preference. More commonly used in ethanol research (Tabakoff & Hoffman, 2000), two-bottle choice procedures can be used to measure the voluntary intake of other drugs, including MA (Eastwood & Phillips, 2014a; Murphy et al., 2021; Wheeler et al., 2009; Ye, Pozos, Phillips, & Izquierdo, 2014). Injection and smoking are the most common forms of MA consumption in humans, but oral administration is frequently the preferred method (McKetin et al., 2006; Simon et al., 2002). Furthermore, drinking is a natural behavior in mice, and does not require training. Two-bottle choice is also a fairly inexpensive method of measuring drug intake, and an entire drinking procedure can be performed in a

relatively short amount of time. Compared to time, space, and equipment requirements of operant self-administration, two-bottle choice is a preferable option for selective breeding (discussed later). Operant self-administration is also more disruptive to the subject. Surgeries are stressful and can be dangerous. For operant-self administration, mice have to be removed from their home cages. For two-bottle choice drinking, mice have continuous access to drug, water, and food, so they can satiate thirst and hunger at will and sample and consume drug on a voluntary basis. Two-bottle choice procedures are minimally invasive, reducing stress on subjects.

In two-bottle choice procedures, animals are offered two bottles, one containing water and one containing the drug of interest. The drug bottle is often offered for 24 hours, but shorter periods can be selected based on the goals of the study. For instance, our typical two-bottle choice procedure for MA drinking consists of 18 hours of access to MA, and 6 hours of access to just water (Shabani, McKinnon, Reed, Cunningham, & Phillips, 2011; Wheeler et al., 2009). This was originally a method chosen, based on preliminary data of a colleague (John K. Belknap, personal communication) to reduce possible MA-induced anorectic effects that could result in weight loss (Wheeler et al., 2009). However, subsequent studies revealed that mice voluntarily consume more MA under these intermittent access conditions than when given 24-hour access (Stafford, Reed, & Phillips, 2020), suggesting that intermittent abstinence periods may lead to higher intake. Drug access can be shortened even further, as was done in Eastwood & Phillips (2014) in order to capture the effects of pretreatment with a drug before that drug is metabolized or the drug effect wanes (Eastwood & Phillips, 2014a).

There are a few noteworthy limitations to two-bottle choice procedures. The taste of the solution in the drug bottle is a primary concern. Mice are neophobic, particularly to tastes (Kronenberger & Médioni, 1985), so may be cautious when presented with a strongly flavored solution. Mice can adapt to tastes given time (Mura, Taruno, Yagi, Yokota, & Hayashi, 2018), but will largely show a preference for water over a bitter solution even after repeated exposures

(Mura et al., 2018). When testing more than one population of mice, a difference in ability to perceive tastes, or innate differences in preference, might obscure results. This can be addressed by also measuring the separate populations' preference for tastes. For instance, when characterizing our selectively bred MA drinking lines (discussed below), we measured their intake and preference for sweet, salty, and bitter tastes and found no significant differences (Shabani et al., 2011; Wheeler et al., 2009). MA has a bitter flavor (https://nida.nih.gov/publications/drugfacts/methamphetamine), so addressing taste preferences is particularly important. Since the lines did not differ in their consumption of these novel tastants, it is unlikely that taste is affecting their two-bottle choice drinking behaviors.

Conditioned taste aversion (CTA)

CTA is a particularly useful method for assessing sensitivity to the perceived aversive effects of a drug. Drug exposure (and necessarily all the interoceptive effects accompanying it) is paired with a non-psychoactive unique taste, often salty or sweet. If those interoceptive effects are aversive, mice will decrease consumption of the unique tasting solution upon subsequent presentations (Davis & Riley, 2010; Sanchis-Segura & Spanagel, 2006; Welzl, D'Adamo, & Lipp, 2001). Tastant consumption becomes a measure of how aversive an animal finds the drug effect(s), thus a greater reduction in tastant intake signals greater sensitivity to the aversive effects of the drug.

CTA procedures are simple to implement and can produce effects after only 1 or 2 drug-tastant pairings (Harkness et al., 2015; Wheeler et al., 2009). Mice may be given access to the unique tastant prior to any drug pairings to account for neophobia of the tastant, but this is not strictly necessary and CTA is observed with and without acclimation to the tastant (Barkley-Levenson, Cunningham, Smitasin, & Crabbe, 2015; Broadbent, Muccino, & Cunningham, 2002). Animals are typically water deprived to ensure proper motivation to consume the tastant during a particular time period.

Conditioned place preference/aversion

In CTA studies, the tastant serves as a conditioned stimulus (CS) to the drug effect's unconditioned stimulus (US). Other cues can serve as a CS though. In conditioned place preference (CPP) and conditioned place aversion (CPA) procedures, the CS is a specific context (Cunningham, Gremel, & Groblewski, 2006). In this procedure, the drug of interest is paired with one unique context (CS+) and vehicle is paired with a another unique context (CS-) (Cunningham et al., 2006). Mice are restricted to the appropriate context during these pairings. After multiple pairings, animals are given a preference test, in which they are presented with both contexts (CS+ and CS-), and the time spent in the respective contexts is measured. Following conditioning, more time spent in the CS+ context is considered CPP, and more time spent in the CS- context is considered CPA. For CPP procedures, forward conditioning is employed and the drug or vehicle is given immediately before CS exposure (Cunningham et al., 2006). When the drug is given following CS exposure, reverse conditioning, this typically produces CPA (Cunningham et al., 2006; Cunningham, Okorn, & Howard, 1997); however, CPA can also be seen using forward conditioning (Shab et al., 2020; Shabani et al., 2011). The magnitude of the difference between the time spent in the CS+ vs the CS- signals the degree of aversion or preference.

Measures of acute sensitivity

An animal's immediate response to a drug can serve as a broad measure of sensitivity to the drug. While not a behavioral effect, most drugs produce a temperature response in rodents, offering a simple and rapid approach to assessing drug effects. Ethanol produces hypothermia through vasodilation and disruptions to the central nervous system (Kalant & Lê, 1983). Opioids can disrupt autonomic thermoregulation, leading to reduced core body temperature (Adler, Geller, Rosow, & Cochin, 1988). Morphine can also activate mu-opioid receptors (OPRM1) in the hypothalamus, producing hyperthermia (Cintron-Colon et al., 2019; Handler, Geller, Adler, Handler, & Adler, 1992; Xin, Geller, & Adler, 1997). In rats and mice, higher doses of morphine produce hypothermia though via kappa opioid receptors, OPRM1

receptors in the periphery, and as yet undetermined mechanisms (Baker & Meert, 2002; Cintron-Colon et al., 2019; Handler et al., 1992; Rosow, Miller, Pelikan, & Cochin, 1980; Xin et al., 1997). The doses required to induce hypothermia in rats are > 20 mg/kg (Chen, Geller, Kim DeRiel, Liu-Chen, & Adler, 1996; Geller, Hawk, Keinath, Tallarida, & Adler, 1983; Rawls & Benamar, 2011), but in mice doses > 10m/kg induce hypothermia (Baker & Meert, 2002; Rosow et al., 1980). Hyperthermic effects of MA and amphetamine-like substances are commonly studied (Matsumoto et al., 2014; Sprague, Riley, & Mills, 2018), but at certain doses and time points after administration, these drugs can also induce profound hypothermia (Harkness et al., 2015; Mootz, Miner, & Phillips, 2020; Shokry, Shields, Callanan, Ma, & Tao, 2019; Xue, Siemian, Zhu, Blough, & Li, 2019).

Drugs also frequently enhance or depress locomotor activity. These effects are time and dose dependent. For instance, in rats, doses of morphine ≤ 10 mg/kg increase locomotor activity, but doses much higher than 10 mg/kg produce an initial decrease in locomotor activity followed by an increase in activity (Babbini & Davis, 1972; Craft, Clark, Hart, & Pinckney, 2006). In mice, doses as high as 32 mg/kg are stimulating (Belknap et al., 1998), but this depends heavily on the mouse strain. In C57BL/6J (B6) mice, morphine increases locomotor stimulation with increased morphine doses, whereas DBA/2J (D2) mice never display morphine-induced locomotor stimulation (Belknap, Richards, O'toole, Helms, & Phillips, 1997). Ethanol is stimulating at low doses, and depressing at higher doses. In mice, doses ≤ 2 g/kg tend to increase locomotor activity, and doses above that tend to depress activity (Cunningham, 2014, 2019; Frye & Breese, 1981; Phillips, Huson, Gwiazdon, Burkhart-Kasch, & Shen, 1995). While mouse strains vary for ethanol-induced locomotor effects (Cunningham, 2014, 2019), they are not as profound as the strain effects observed with morphine. As a central nervous system stimulant, MA reliably produces an increase in locomotor activity in a dose-dependent manner in both mice and rats (Bevins & Peterson, 2004; Scibelli et al., 2011; Shabani et al., 2011). It is important to account for locomotor activity changes due to drugs. Heavy sedation, or stimulation

to the point of stereotypy, may prevent an animal from performing a behavior of interest. They may cease pressing a lever or drinking from a tube. Stimulation could also cause an animal to arbitrarily perform an action, such as pressing a lever.

Genetic Methods: Quantitative trait locus mapping

Despite being referred to, colloquially, as a single disease, addiction is a complex trait comprising a set of behaviors caused by any number of genetic variables, environmental factors, and gene by gene and gene x environment interactions. The behavioral assays discussed above are intended to select and distill addiction traits to better understand their origins. However, each of these traits is complex, and under the control of independent and interactive gene and environmental effects (Palmer & Phillips, 2002). Quantitative trait locus (QTL) mapping is one method to begin resolving genetic risk factors (Palmer & Phillips, 2002). Specific genes are rarely identified by an initial analysis; rather a region is identified that contains a gene or genes responsible for variability in a quantitative trait (Gonzales & Palmer, 2014; Lebowitz, Soller, & Beckmann, 1987; Palmer & Phillips, 2002). In QTL mapping, known genetic markers are correlated with a trait of interest (Mackay, 2001; Palmer & Phillips, 2002). The genetic markers can be any known polymorphisms (variations of a particular DNA sequence) between two or more populations. While statistically controlling for the number of comparisons made, correlations between each genetic marker and the phenotype of interest are considered for significance (Belknap et al., 2001; Berrettini, Ferraro, Alexander, Buchberg, & Vogel, 1994; Palmer & Phillips, 2002). The precision of estimates of the position of a QTL, the mapping resolution, increases with more markers (Belknap et al., 2001; Palmer & Phillips, 2002). Once the QTL has been localized and refined to a suitably small chromosomal region, specific gene contributions can be probed. There are many strategies for this (Homanics, 2002; Palmer & Phillips, 2002), and they will not be covered extensively here. Knock-in (KI) and knockout (KO) mouse lines can be developed by inserting a specific gene variant (KI) or deleting a gene (KO) of interest (Homanics, 2002; Palmer & Phillips, 2002). Conditional

knockout mice permit control over the location and the timing of gene manipulation (Navabpour, Kwapis, & Jarome, 2020), and are pivotal in defining the exact role a candidate gene plays in a particular phenotype.

Populations of study for QTL mapping

A successful QTL mapping population must have a heritable phenotype, and must be genetically heterogeneous. Traditional inbred strains are the backbone on which QTL mapping populations are built, and are the common progenitors of mapping populations (Mackay, 2001; Palmer & Phillips, 2002). Panels of inbred strains have been used to identify phenotypic differences among strains (Belknap et al., 1998; Belknap, Crabbe, Riggan, & O'Toole, 1993; J K Belknap, Crabbe, & Young, 1993; Cunningham, 2019). These panels can also be used to estimate heritability (H^2) of the phenotype. In other words, they permit an estimate of the proportion of phenotypic variance under genetic control for the population under study. For instance, a panel of 15 inbred mouse strains estimated the H^2 of morphine-induced locomotor activity to be 0.39, and the H^2 of morphine-induced temperature changes to be 0.47 (Belknap et al., 1998). In another panel of inbred strains, mice underwent an ethanol CPA procedure, and H^2 for CPA and ethanol-induced locomotor activity was estimated for each ethanol dose used (2 and 4 g/kg) (Cunningham, 2019). Ethanol-induced activity, and even activity during drug-free habituation sessions, were highly heritable, with H^2 values generally >0.40. H^2 for ethanol CPA at both doses was low though, between 0.06 and 0.14 (Cunningham, 2019). Thus, ethanol CPA would be a poor choice for a phenotype for QTL analysis, but morphine-induced temperature changes would be appropriate.

Crosses of two inbred strains

Breeding two inbred strains together produces an F_1 population. Same-sex individuals are genetically identical, and are heterozygous at all loci (Crabbe, Phillips, Kosobud, & Belknap, 1990; Palmer & Phillips, 2002). Breeding F_1 individuals together creates an F2 population. Each F2 mouse possesses a random combination of the genes possessed by the progenitor strains

(Crabbe et al., 1990). The phenotype of interest can be measured in the F2 population and the subjects genotyped and QTL mapping performed (Palmer & Phillips, 2002). These produce a relatively low-resolution map, so are better suited for initial QTL mapping.

Recombinant inbred strains

F2 mice can be inbred in an identical manner to that used to produce classic inbred strains. When the cross of two F2 mice are bred to homozygosity by brother-sister mating, the result is a recombinant inbred (RI) strain, a population that possesses a unique recombination of the parental strains' genes and is homozygous at all loci (Palmer & Phillips, 2002). Multiple RI strains can be created, producing a panel of RI strains, something akin to clonal populations representing samples of an F2 population. Because every individual within a RI strain can be considered genetically identical, once polymorphism information is available throughout the genotype for each RI strain the strains can be genetically compared without the need to genotype every subject, reducing time and costs (Palmer & Phillips, 2002). RI strains also require additional breeding generations to produce, compared to F2 populations. This reduces linkage disequilibrium, thereby enhancing mapping resolution (Palmer & Phillips, 2002). A good example of the use of a BXD RI panel of strains for mapping is their use to identify QTLs for MA responses (Grisel et al., 1997). The BXD RI strains were created from B6 and D2 progenitors. Responses to MA were measured in 25 BXD RI strains (Grisel et al., 1997). These included stereotyped chewing and climbing, locomotor activity, and body temperature changes. Putative QTLs for one or more responses were found on all chromosomes except for mouse chromosomes 11 and 18 (Grisel et al., 1997). A more recent example is the discovery of a role of a member of the Ras oncogene family, *Rab3b*, in cocaine stimulation (Bubier, Philip, Dickson, Mittleman, & Chesler, 2020; Philip et al., 2010). An early study had analyzed data from a battery of behavioral tests performed in BXD RI strain, which revealed purported QTLs for cocaine-induced locomotor stimulation in mouse chromosomes 4 and 15 (Philip et al., 2010). Bubier et al. (2020) reanalyzed and refined these QTLs, and compared the genes in these QTLs against

lists of genes generated from differential gene expression analyses of BXD RI strains and relevant published experiments (Bubier et al., 2020). *Rab3b* emerged as a leading candidate gene for cocaine stimulation. An important role for *Rab3b* in cocaine stimulation was confirmed with *Rab3b* KO mice, which were significantly more sensitive to cocaine-induced locomotor stimulation and sensitization (Bubier et al., 2020).

Heterogeneous stocks

RI strains developed from two inbred strains are powerful mapping tools, but are limited by the amount of genetic variation present in the chosen strains. Heterogeneous stock (HS) mice bypass this problem, if derived from multiple strains. The collaborative cross (CC) RI strains are derived from the intercrossing of 8 genetically diverse inbred mouse strains (Chesler, 2014; Threadgill, Miller, Churchill, & de Villena, 2011), and from these the diversity outbred (DO) heterogeneous population was created (Chesler, 2014; Svenson et al., 2012). In addition to greater genetic diversity, both populations offer a high number of recombination events, increasing mapping resolution.

Selectively bred lines

Selective breeding is a powerful tool for initial QTL mapping. If a phenotype has a genetic component, then it can be selected for. In this case, by breeding individuals with a shared phenotype together, the offspring are more likely to possess that phenotype. This is the same procedure used in agriculture, livestock, and dog breeding to generate animals and plants with desired traits. If the selective breeding process is repeated, the alleles responsible for the phenotype will concentrate in subsequent generations, and allele frequencies for those genes impacting the trait will increase (Crabbe, 1999; Falconer & Mackay, 1996).

Selective breeding can be performed with any progenitor population that is genetically heterogeneous. F2 populations created from 2 inbred strains are common, but more genetically diverse populations like the DO can also be used (Chesler, 2014; Svenson et al., 2012). Selection can occur bidirectionally or unidirectionally. In bidirectional selective breeding, the

initial heterogeneous population is tested, and the highest and lowest scorers are selected. For unidirectional, selective breeding is performed in only one direction (high or low). For in stance, the High drinking in the dark mouse lines (HDID) were selected for high ethanol consumption during a drinking in the dark procedure, but no "low" line was ever established (Barkley-Levenson & Crabbe, 2014; Barkley-Levenson et al., 2015; Crabbe et al., 2009; Crabbe, Phillips, & Belknap, 2010).

The method for selecting individuals and how to breed is dependent on the breeding strategy. There are 2 breeding strategies that have been most often used. The first and perhaps simplest is individual/mass selection, where the highest scoring males and females are bred together, and/or the lowest scoring males and females are bred together (Crabbe, 1999). If the selection is bidirectional, after breeders are chosen from the initial heterogeneous population, independent high and low lines are established and breeding continues, choosing the highest scoring individuals from the high line offspring for the next generation of breeding, and choos ing the lowest scoring individuals from the low line offspring for the next generation. This typically results in a rapid selection response. However, certain families will contribute more breeders than others, resulting in a higher rate of inbreeding (Crabbe, 1999; Crabbe 2014). Intentionally avoiding related breeding pairs can help reduce this rate (Belknap et al., 1997). Short term selective breeding utilizes mass/individual selection for a limited number of generations (Belknap, Richards, O'toole, Helms, & Phillips1, 1997; Palmer & Phillips, 2002). Genetic drift, the change in frequency of alleles in a population over generations, and/or inbreeding can cause genes unrelated to the trait of interest to inevitably become fixed the longer selection continues, thus limiting generations mitigates that risk (Crabbe, 1999; Falconer & Mackay, 1996; Palmer & Phillips, 2002). This is particularly important when using these lines for QTL analyses, as homozygosity at trait-irrelevant genes can lead to false-positives (Crabbe, 1999; Crabbe, 2018; Palmer & Phillips, 2002). Additionally, reproducing short-term selected lines, using an independent progenitor population (i.e. independent replicates), can help account for trait-

irrelevant gene fixation that occurs in any one replicate (Falconer & Mackay, 1996; Palmer & Phillips, 2002). One must be cautious in interpreting this, however, because genetic drift can result in the chance disappearance of particular alleles from a population, associated with the death of specific individuals or because individuals do not reproduce. Thus, relevant alleles can be lost in one selection replicate and not the other.

The second method is within-family selection. In this method, the most extreme scoring (high or low) male and female from every family are selected for breeding (Crabbe, 1999; Falconer & Mackay, 1996). Again, after the initial choices from the heterogeneous population are made, independent high and low lines are established and selection continues within each line. The rate of selection response is generally slower for this method, but the method has a lower rate of inbreeding than individual/mass selection.

If a selection phenotype is heritable, the mean score for the phenotype will be greater (or lesser) in the offspring, relative to the original population (Crabbe, 1999; Crabbe, 2018). This difference is the response to selection (*R*) (Crabbe, 1999; Crabbe, 2018). The realized R – the trait difference between the last and first generations – can be divided by the cumulative selection differential (*S*) – the difference between the mean score of the selected breeders and the mean score of the population from which they were selected – to estimate the realized heritability (h^2) (Crabbe, 1999; Falconer & Mackay, 1996). Once all trait relevant genes have been fixed, the change in trait scores between generations will plateau. It is possible for heterozygosity to remain at trait-relevant loci, if there is dominance and heterozygotes and homozygotes score similarly for the selection phenotype. If only a few genes are relevant for the selection phenotype, the selection plateau will be reached fairly quickly.

Selected mouse lines have been developed for addiction research. Some of the best examples come from ethanol research. Two different alcohol intake phenotypes have been selectively bred in mice. Belknap et al. (1997) used a B6D2F2 population to develop ethanol high preferring and low preferring short-term selected lines, characterized by the ratio of water

to ethanol they consumed in a two-bottle choice paradigm (Belknap et al., 1997). By the first generation, differences in ethanol preference were observable. For several genetic markers this was accompanied by a rapid change in allele frequency, and one allele became fixed in the low preference line by the 4th generation. Another set of alcohol high preferring and low preferring mice was generated using an HS population created by systematically crossing 8 inbred mouse strains (Grahame, Li, & Lumeng, 1999). Mice were selected based on g/kg of ethanol consumed. This set first utilized a within-family selection approach, but little selection response was observed (Grahame et al., 1999). After the 3rd generation, within-family selection was abandoned, and mass selection was implemented. Mass selection produced a robust selection response (Grahame et al., 1999). Long-term, unidirectional selective breeding produced two replicates of HDID mouse lines from a population of HS/Npt mice, produced from the systematic crossing of 8 inbred strains (Barkley-Levenson & Crabbe, 2014; Crabbe et al., 2009, 2010). While they will not be discussed in detail here, the number of other ethanol-related phenotypes that have successfully been subjected to selective breeding highlights the versatility of selective breeding strategies. Short-term selective breeding successfully produced lines of mice for high and low ethanol-induced CTA (Phillips et al., 2005); mouse lines have been selectively bred for susceptibility (COLD) or resistance (HOT) to the hypothermic effects of ethanol (Crabbe, Kosobud, Tam, Young, & Deutsch, 1987; Crabbe, Belknap, & Buck, 1994; Feller & Crabbe, 1991); the long-sleep and short-sleep lines were bred for sensitivity to the sedating effects of ethanol (Crabbe et al., 1994; McClearn & Kakihana R, 1981).

In addition to the MA drinking lines described in the next section, selected lines have been developed for other MA-related traits. B6D2F2 populations have been used in short-term selection procedures to develop lines of mice with high and low sensitivity to MA-induced stereotyped chewing (Atkins, Helms, O'Toole, & Belknap, 2001), high and low MA-induced locomotor activation (Kamens et al., 2005), and high and low MA-induced locomotor

sensitization (Scibelli et al., 2011). The lines described here are just a sample of a larger array of differentially MA-sensitive lines that have been created.

The MA drinking lines

Our lab has utilized short-term mass selection to generate lines of mice selectively bred for high (MAHDR) and low (MALDR) voluntary MA drinking. These selected lines, collectively called the MA drinking or MADR lines, were derived from a B6D2 F2 founder population (Wheeler et al., 2009). Selection was based on voluntary MA intake in an oral two-bottle choice procedure. Other forms of self-administration, such as intravenous self-administration, are technically challenging and require specialized equipment, making them unsuitable for selective breeding procedures that require the testing of large numbers of mice. The MADR lines have been independently replicated 5 times, at a 2-year interval, with nearly identical results for each replicate (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009).

The MADR lines were selectively bred based on MA intake from a 40 mg/L MA concentration solution; MA was dissolved in tap water and offered in a two-bottle choice test vs. unadulterated tap water (Wheeler et al., 2009). By the first selection generation (S1) the lines differed significantly in MA intake and preference for MA over water (Shabani et al., 2011; Wheeler et al., 2009). By the S2-S3 generation, depending on the selection replicate, MALDR line mice consumed virtually no MA, whereas MAHDR line mice consumed 6-8 mg/kg MA over an 18-hour period. MAHDR line mice have also been shown to self-administer MA in an operant conditioning task (Shabani, Dobbs, et al., 2012). When MA concentrations were increased, MAHDR line mice consumed progressively increasing quantities of MA (Shabani et al., 2016). When the number of bottles containing MA was increased to a higher ratio than 1:1 vs. water, MAHDR line mice consumed upwards of 30 mg/kg MA (Shabani et al., 2016), compared to the 6-8 mg/kg they consume when the ratio is 1:1 (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009).. Under the higher ratio conditions, MALDR line mice consumed approximately 5 mg/kg at most (Shabani et al., 2016).

Most of the response to selection, as measured by MA intake, occurred within the first two selection generations (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). By the 4th selection generation, but as early as S2 depending on the selection replicate, response to selection ceases (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). This rapid differentiation in MA drinking, is consistent with only a few genes playing a role in level of MA intake (Crabbe, 1999; Falconer & Mackay, 1996). This is strongly supported by the reproducibility of these results in independent selection replicates.

Glutamate systems in MADR line mice

Because glutamate dysregulation is strongly implicated in addiction (Kalivas, 2009), the glutamate systems of MADR line mice have been of interest. Research performed in the laboratory of a collaborator of Dr. Phillips measured glutamate-related proteins and glutamate levels in brain tissue from the MADR lines. Within the NAcc core and shell, MAHDR line mice had greater levels of mGluR5, and the scaffolding protein Homer2a/b than MALDR line mice. Within the NAcc core alone they had decreased levels of EAAT3 and the scaffolding protein Homer1b/c (Szumlinski et al., 2017). MAHDR line mice also had higher baseline NAcc glutamate and a greater increase in NAcc glutamate following a challenge dose of MA (Szumlinski et al., 2017). Within the PFC, MAHDR line mice had greater levels of Homer2a/b and mGluR2, when compared to MALDR line mice (Lominac et al., 2016). Also within the PFC, MAHDR line mice had elevated extracellular glutamate, but exhibited a decrease in PFC glutamate from a challenge dose of MA not observed in MALDR line mice (Lominac et al., 2016). Using RNA sequencing (RNA-seq) in the 3rd selection replicate of MADR lines, an analysis assessing how selection impacts the differences gene co-expression implicated a number of glutamate-related genes in the PFC (Hitzemann et al., 2019). These included the metabotropic glutamate receptors 1 and 5, the scaffolding protein Homer2, and AMPA receptor subunits.

Aversive effects of MA

MAHDR line mice have a remarkably low sensitivity to the aversive effects of MA, contrasted by the MALDR line's profound sensitivity to these aversive effects. In 2 separate selection replicates of MADR lines, MALDR line mice developed a rapid and robust CTA to 1 and 2 mg/kg of MA, whereas MAHDR line mice were completely resistant to MA-induced CTA at doses up to 4 mg/kg (Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). When a reverse conditioning procedure was employed to induce CPA, MALDR mice developed CPA to a lower dose of MA than MAHDR line mice (2 mg/kg vs 4 mg/kg) (Shabani, McKinnon, et al., 2012). When forward conditioning was employed for a CPP procedure, MAHDR line developed CPP to 0.5, 2, and 4 mg/kg doses of MA whereas MALDR line mice did not when both lines were tested in a drug-free test (Shabani et al., 2011). In the drug-present test, MALDR line mice displayed CPA, rather than CPP, to 0.5, 2, and 4 mg/kg doses of MA, and MAHDR line mice displayed CPP to the 0.5 mg/kg MA dose, and no conditioned aversion or preference for the other doses (Shabani et al., 2011).

CTA and CPA do not reveal anything about the specific effects of MA that MALDR line mice perceive as aversive. However, studying a physiological response of MADR mice led to the hypothesis that MA-induced temperature changes are involved. We have consistently found a profound difference in the thermal effects of MA in the MADR lines. The MALDR line becomes markedly hypothermic to MA, whereas the MAHDR line either shows no thermal response to MA, or hyperthermia (Harkness et al., 2015). Studies into hypothermic effects of other drugs support a link between temperature response and drug aversion. Lithium chloride (LiCl) and ethanol both produce hypothermia and CPA (Cunningham, Hawks, & Niehus, 1988; Cunningham & Niehus, 1993). Raising the ambient temperature of the experimental room, and consequently core body temperatures of mice, attenuates both LiCl and ethanol induced CPA (Cunningham et al., 1988; Cunningham & Niehus, 1993). This connection to drug aversion fits within a larger body of evidence indicating an inverse relationship between drug-induced hypothermia and drug intake, such that the greater the hypothermic response, the lower the

drug intake. When rat lines were selectively bred for high vs low ethanol intake, the high intake line (alcohol accepting; AA) was also less sensitive to ethanol-induced hypothermia than the low intake line (alcohol non-accepting; ANA) (Sinclair, Lê, & Kiianmaa, 1989). We can also see this trend in the MADR lines' progenitor strains, the B6 and D2 inbred strains. D2 mice consume less morphine (Belknap, Crabbe, Riggan, et al., 1993; Doyle et al., 2008, 2014), and are also more sensitive to the hypothermic effects of morphine than B6 mice (Belknap, Noordewier, & Lamé, 1989; Takamura Muraki & Ryuichi Kato, 1987).

The MADR lines also clearly align with this inverse hypothermia/drug intake phenomenon. There are two potential explanations for this. First, reductions in body temperature might increase the period during which negative associations can be formed (Christianson, Misanin, Anderson, & Hinderliter, 2005; Misanin et al., 2002; Misanin, Wilson, Schwarz, Tuschak, & Hinderliter, 1998). If this holds true for MA-induced hypothermia, then both MADR lines might experience similar aversive effects, but the association between those effects and MA intake might be significantly stronger in MALDR line mice. Another possibility is that hypothermia itself is aversive. This is certainly possible given maintenance of a specific body temperature is necessary for survival and drastic reductions in core body temperature provoke stress responses (Bańka, Teresiński, Buszewicz, & Mądro, 2013; Okuda, Miyazaki, & Kuriyama, 1986; Shida et al., 2020). Raising core body temperature, as was done with LiCl and ethanol, cannot differentiate between these possibilities however. Upon initial presentation with MA, MALDR and MAHDR mice consume comparable amounts (Eastwood, Barkley-Levenson, & Phillips, 2014; Shabani, Dobbs, et al., 2012). In a 4-hour period, MALDR line mice consume approximately 0.3 – 0.4 mg/kg MA. This may not be enough to even produce hypothermia. However, MALDR line mice become hypothermic after a 1 mg/kg bolus injection of MA, decreasing core body temperature upwards of 2-degree Celsius (Harkness et al., 2015).

Opioid receptors and opioids in MADR line mice

A QTL analysis identified a region at the proximal end of mouse chromosome 10 with a 10-40 Mb interval, which accounts for at least 60% of the genetic variance in MA drinking between the MADR lines (Belknap et al., 2013). This interval contains approximately 200 protein-coding genes, including *Oprm1* (at 6.75 Mb) and *Taar1* (at 23.9 Mb). *Oprm1* was a promising candidate gene for a number of reasons. First, there were already known differences in opioid receptors and opioid intake between the MADR progenitors, the B6 and D2 inbred strains. D2 mice freely consume less morphine than B6 mice (Belknap, Crabbe, Riggan, et al., 1993). A major QTL on mouse chromosome 10, *Mop2*, has been identified for morphine intake differences between B6 and D2 mice (Berrettini et al., 1994; Doyle et al., 2008; Ferraro et al., 2005), and it encompasses *Oprm1*. There are also known polymorphisms in the *Oprm1* promotor regions of B6 and D2 mice (Doyle et al., 2006).

Second, investigations using the MADR lines indicated a role of opioid receptors. When MADR line mice were tested for sensitivity to opioids, MALDR line mice displayed greater locomotor stimulation to morphine and fentanyl (Eastwood & Phillips, 2014a). Both these drugs are OPRM1 agonists. MADR line mice display morphine-drinking phenotypes similar to their progenitor strains, the D2 and B6 mice. MAHDR line mice and D2 mice consume less morphine than B6 mice and MALDR line mice (Eastwood & Phillips, 2014a). This suggests MAHDR line mice possess alleles contributed by D2 mice that limit morphine intake. Genotyping from three replicate sets of MADR lines supported this. 72% of MAHDR line mice were homozygous for the D2-*Oprm1* allele, and 68% of MALDR line mice were homozygous for the B6-*Oprm1* allele (Eastwood, Eshleman, Janowsky, & Phillips, 2018). There is also evidence that opioids play a part in MA intake in MAHDR line mice. While naltrexone was not able to alter MA drinking in MAHDR line mice, buprenorphine, an OPRM1 partial agonist, significantly decreased MA intake (Eastwood & Phillips, 2014a). Naltrexone is a full OPRM1 antagonist, whereas buprenorphine partially activates OPRM1 at lower doses but acts as an antagonist at higher doses (Huang, Kehner, Cowan, & Liu-Chen, 2001; Leander, 1988; Olson, Duron, Womer, Fell, & Streicher,

2019; Virk, Arttamangkul, Birdsong, & Williams, 2009). Finally, there are OPRM1 expression differences between the MADR lines. OPRM1 density is > 2x greater in the mPFC of MALDR line mice than MAHDR line mice (Eastwood et al., 2018). Increased mPFC OPRM1 density or sensitivity enhances impulsive behavior, but most of this work has been performed in eating studies (Baldo, 2016). In both humans and rodents, amphetamine promotes the release of opioids in the mPFC (Colasanti et al., 2012). OPRM1 seems to play an important role in reward value and decision making, particularly in the PFC, VTA, and NAcc (Baldo, 2016; Carr & Sesack, 2000; Castro & Berridge, 2017; Chartoff & Connery, 2014; van Steenbergen, Eikemo, & Leknes, 2019). How increased OPRM1 density in MALDR line mice might modify their MA intake requires more investigation, and the role of the opioid system in non-opioid drug use is still largely unknown. However, endogenous and exogenous opioids reduce glutamatergic signaling from the mPFC to the NAcc and VTA (Baldo, 2016; Carr & Sesack, 2000; Castro & Berridge, 2017; Chartoff & Connery, 2014; van Steenbergen et al., 2019), and these projections are important for drug-motivated behaviors (Kalivas, 2009). Complicating things further, there are OPRM1-mediated hedonic "hotspots" and "coldspots" in the orbitofrontal cortex and insula, at which the OPRM1 agonist DAMGO increased or decreased sucrose reward, respectively (Castro & Berridge, 2017). Thus, the neuronal populations expressing OPRM1 in MALDR line mice could impact their response to MA.

Finally, studies in humans also supported *Oprm1* as a candidate gene. Human *OPRM1* variants are associated with alcohol (Schinka et al., 2002) and cocaine dependence (Hoehe et al., 2000). *OPRM1* variants mediate euphoric effects of MA in humans (Dlugos et al., 2011) and MA-induced psychosis (Ide et al., 2006). Drugs targeting the opioid system can reduce subjective effects and self-administration of stimulants. For instance, the OPRM1 antagonist naltrexone attenuates the subjective effects of amphetamine in people addicted and not addicted to amphetamine (Jayaram-Lindström et al., 2008; Jayaram-Lindström, Wennberg, Hurd, & Franck, 2004). The partial OPRM1 agonist buprenorphine can decrease cocaine self-

administration in monkeys (Mello, Mendelson, Bree, & Lukas, 1989), and among patients being treated for opioid addiction, buprenorphine can reduce concurrent cocaine use (Kosten, Kleber, & Morgan, 1989).

Finer mapping in existing chromosome 10 congenic strains derived from B6 and D2 mice ruled *Oprm1* out as the QTG for differential MA intake risk in the MADR lines (Doyle et al., 2008, 2014; Eastwood et al., 2018); however, it was a regulatory hub for the top-ranked transcription factor network for MA intake risk (Belknap et al., 2013).

The research into the MADR lines' opioid systems revealed the relevance of opioids to their MA drinking behavior. Taken together, it was possible that *Oprm1* was an important gene for MA intake differences between the MADR lines, and although it proved not to be a QTG it seemed likely that the opioid system was at least involved in MA intake in these lines. This was supported by work done after the initiation of this dissertation. *Taar1* will be discussed shortly, but *Oprm1* and *Taar1* have interactive effects on MA intake and hypothermia, such that the effects of *Taar1* genotype were synergistically enhanced by *Oprm1* genotype (Stafford et al., 2019).

Taar1

This left *Taar1* as the most promising QTG candidate in the chromosome 10 QTL region. Recall that amphetamine and MA are TAAR1 agonists (Bunzow et al., 2001; Wolinsky et al., 2007). MAHDR and D2 mice have similar MA drinking profiles, as do MALDR and B6 mice (Eastwood & Phillips, 2014b; Reed et al., 2017). This suggested that MAHDR line mice are genetically more similar to D2 mice, at least on mouse chromosome 10, whereas MALDR line mice are more genetically similar to B6 mice. D2 mice possess a non-synonymous single-nucleotide polymorphism (SNP) at position 229 (C229A) (Sanger Mouse Genomes Project SNP Keane et al., 2011; Sanger, 2014; Yalcin et al., 2011) unique to the D2 strain. This SNP results in a proline to threonine substitution (P77T) (Harkness et al., 2015; Keane et al., 2011; Shi et al., 2016; Yalcin et al., 2011), and is unique to DBA/2 mice from the Jackson Laboratory, as it is

absent in DBA/2 mice from other suppliers (Reed et al., 2017) and absent from all 28 inbred mouse strains that have been sequenced (Keane et al., 2011; Shi et al., 2016) We found that by the 4th selection generation, MAHDR line mice are homozygous for this SNP, now called *Taar1^{m1J}*, whereas MALDR line mice are either heterozygous or homozygous for the reference B6 allele (Harkness et al., 2015; Mootz, Miner, & Phillips, 2020).

The nucleotide substitution present in *Taar1^{m1J}* occurs in the second transmembrane domain, near a predicted binding pocket stabilizing residue (Reese et al., 2014). Proline provides particular molecular dynamics that are often important for proper receptor function (Mazna et al., 2008; Reiersen & Rees, 2001; Sansom & Weinstein, 2000; T. W. Schwartz, Frimurer, Holst, Rosenkilde, & Elling, 2006; Van Arnam, Lester, & Dougherty, 2011). *Taar1* knockout (*Taar1$^{-/-}$*) mice are more sensitive to MA-induced locomotor stimulation (Achat-Mendes, Lynch, Sullivan, Vallender, & Miller, 2012; Lindemann et al., 2008; Wolinsky et al., 2007), signifying TAAR1 function mediates certain MA effects. This raised the possibility that *Taar1^{m1J}* encodes a nonfunctional receptor. Our lab further characterized *Taar1$^{-/-}$* mice for MA-related traits, and found that *Taar1$^{-/-}$* and MAHDR line mice share similar phenotypes: they freely consume MA, are resistant to MA-induced CTA, and are resistant to MA-induced hypothermia (Harkness et al., 2015; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). The similar phenotypes between MAHDR line mice and *Taar1$^{-/-}$* mice supported the hypothesis that *Taar1^{m1J}* may express a receptor with altered or absent function. Indeed, when MA or the endogenous trace amines phenethylamine and tyramine were applied to HEK293 cells transfected with the D2-like *Taar1^{m1J}* isoform, there was no cAMP response for the expressed receptors (Harkness et al., 2015). This is compared to cells transfected with the B6-like *Taar1$^{+/+}$* isoform, which did produce a TAAR1 receptor cAMP response when MA was applied (Harkness et al., 2015). Differences in receptor expression are not likely to be the cause of any of the behavioral or activity differences since cells transfected with *Taar1^{m1J}* still robustly expressed a receptor (Harkness et al., 2015). At the time, this seemed to confirm that MAHDR line mice

expressed a non-functional TAAR1. However, cAMP is an indirect measure of function. As was noted earlier, TAAR1 signals through a $G\alpha_s$- and a $G\alpha_{13}$-mediated pathway (Underhill et al., 2020, 2019). Only the $G\alpha_s$-mediated signaling pathway requires cAMP, leaving open the possibility that MAHDR line mice possess at least a partially functional TAAR1. The Taar1-$G\alpha_{13}$-mediated pathway has yet to be explored.

Taar1 was confirmed as a QTG through experiments employing CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat (CRISPR) CRISPR-associated protein 9) technology to swap $Taar1^{m1J}$ in MAHDR line mice with the reference allele, $Taar1^{+}$. The CRISPR-Cas system is a part of a microbial immune system that adapts to novel viruses and plasmids by integrating foreign DNA (Barrangou et al., 2007). This system has been exploited for genome editing, allowing for precise incorporation of desired DNA sequences into a host genome (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Gasiunas & Siksnys, 2013; Shalem, Sanjana, & Zhang, 2015). Knock-in of $Taar1^{+}$ in MAHDR line mice virtually abolished MA intake, conferred MA-induced hypothermia (Stafford et al., 2019) and CTA (Phillips et al., 2021), and reduced MA-induced conditioned place-preference (Phillips et al., 2021). These studies confirmed *Taar1* as a QTG for MA intake differences between the MADR lines, and demonstrated a key role of *Taar1* genotype in the MA-related phenotypes observed in the MADR lines.

Hypotheses and experimental aims

The MADR lines offer a promising model for studying risk factors for MA addiction, and mechanisms that might protect against it. The current project aimed to investigate phenotypes conferred by selective breeding for MA intake. In Chapter 2, I report data determining the binding ability of the D2-TAAR1, the receptor encoded by $Taar1^{m1J}$. The discovery of *Taar1* as a QTG for MA intake differences between the MADR lines is a key component in understanding risk and protection in these lines, but a number of important questions remained. We did not know the direct consequences of the $Taar1^{m1J}$ mutation. The lack of cAMP response to TAAR1

agonists in D2-TAAR1 expressing cells suggests the mutant receptor is non-functional (Harkness et al., 2015), but at what part of the signaling process functionality is disrupted is not known. Since the initial studies into D2-TAAR1 function, TAAR1 signaling pathways have been more fully examined (Underhill et al., 2020, 2019). To date, 2 have been identified, but only one is known to require cAMP. The possibility remained that the TAAR1 expressed in MAHDR line mice, the D2-TAAR1, can still engage this signaling pathway and produce cellular effects. This is only possible, however, if D2-TAAR1 can bind ligand. In the event that it cannot, no signaling pathways could be engaged by MA and it would be a truly nonfunctional receptor (Harkness et al., 2015). I used a radiolabeled TAAR1 ligand, [3H]RO5166017, to probe the ability of D2-TAAR1 to bind ligand.

In Chapter 3, I present data on the intersection of genetic risk for MA intake with thermal responses to other addictive drugs. We now have strong evidence that TAAR1 mediates MA-induced hypothermia (Harkness et al., 2015; Stafford et al., 2019). As hypothermia is a potential explanation for the low MA intake in MALDR line mice, exploring drug-induced hypothermia could further indicate protective mechanisms against high MA intake. I tested 3 addictive drugs, 3,4-Methylenedioxymethamphetamine (MDMA), cocaine, and morphine. Like MA, MDMA is an amphetamine-like substance that activates TAAR1. Since TAAR1 activation mediates MA hypothermia in the MADR lines, I hypothesized that MDMA would induce temperature changes similar to those observed for MA (Harkness et al., 2015). Cocaine is a stimulant that does not activate TAAR1. MADR line mice do not differ in cocaine-conditioned reward and aversion (Gubner, Reed, McKinnon, & Phillips, 2013). Because of this, I hypothesized that cocaine would produce similar temperature changes in both MADR lines. While morphine does not activate TAAR1, MAHDR line mice consume more morphine than MALDR line mice (Eastwood & Phillips, 2014a). If the inverse relationship between drug-induced temperature changes and drug intake holds true, then MAHDR line mice should be more sensitive to the hypothermic effects of morphine. *Oprm1* may not be responsible for the MA intake differences in the MADR

lines, but lies close to *Taar1*. Because of this, *Taar1* and *Oprm1* alleles from the same progenitor strain may be inherited together. Functional differences between the B6 and D2 OPRM1 could explain any differences in opioid sensitivity and intake between the MADR lines. I hypothesized that *Oprm1* and *Taar1* are genetically linked in the MADR mice, and that it is *Oprm1* genotype, not *Taar1* genotype, that determines responses to morphine. To examine this, I genotyped all of the MADR mice treated with morphine, and performed morphine hypothermia experiments in a set of BXD RI mice in which *Oprm1* and *Taar1* linkage had been broken, thereby investigating the independent and interactive effects of the 2 genes. Finally, I wanted to examine buprenorphine, an ORPM1 partial agonist that attenuates the acquisition of MA intake in MAHDR line mice (Eastwood & Phillips, 2014a). Since morphine induces substantial hypothermia in MAHDR lines, I hypothesized that buprenorphine would as well. If hypothermia is the effect limiting MA intake in MALDR line mice, then buprenorphine-induced hypothermia could be limiting MA intake of MAHDR line mice. I also hypothesized that, because MALDR line mice are less sensitive to the hypothermic effects of morphine, they would also be less sensitive to the hypothermic effects of buprenorphine.

Finally, in Chapter 4, I present research investigating the role of glutamate in the development and maintenance of MA drinking in MAHDR line mice. I hypothesized that pharmacologically targeting proteins that are known to be perturbed by drug exposure would reduce MA intake in MAHDR line mice. I tested NAC, the mGluR2 positive allosteric modulator biphenylindanone A (BINA), and 2 mGluR5 negative allosteric modulators, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) and N-(5-Fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (VU0424238). All drugs were tested for their ability to attenuate the acquisition of MA drinking. The mGluR5 allosteric modulators were also tested for their ability to alter established MA drinking. Additionally, I tested the effects of voluntary oral NAC administration on the acquisition of MA intake. The final component of this chapter reports glutamate-related protein levels in the NAcc of MAHDR and MALDR line mice. I measured

proteins (mGluR5, Homer2a/b, EAAT3, and EAAT2) that had been measured in an earlier selection replicate (Szumlinski et al., 2017), as well as additional proteins (x_c^- and VGLUT1) that have not been explored in these lines.

Chapter 2:

Functional effects of a single nucleotide polymorphism on the trace amine-associated receptor 1

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Abstract

The methamphetamine (MA) drinking lines (MADR) were developed to study the genetic and biological underpinning of high (MAHDR) and low (MALDR) MA intake. A quantitative trait locus (QTL) analysis identified a region on mouse chromosome 10 accounting for the majority of the variance in MA intake between the MADR lines. The trace amine-associated receptor 1 gene (*Taar1*) lies within the confidence interval of this QTL, and subsequent studies have identified *Taar1* as a quantitative trait gene for MA intake differences in the MADR lines. MAHDR line mice are homozygous for a *Taar1* allele ($Taar1^{m1J}$) contributed by one of the progenitor strains, the DBA/2J (D2) inbred mouse strain. This allele has a single nucleotide polymorphism (SNP) in *Taar1* resulting in a proline to threonine substitution in the second transmembrane domain. The resulting receptor, mTAAR1-D2, does not produce a cAMP response in the presence of agonists. MALDR line mice are never homozygous for $Taar1^{m1J}$; instead they possess at least one copy of the reference C57BL/6J (B6) allele and express the B6-like receptor, mTAAR1-B6. This receptor appears fully functional by all measures. This difference in functionality is proposed as a primary cause for the MA intake differences between the lines. However, how $Taar1^{m1J}$ disrupts receptor function is not known. Here, the consequences of $Taar1^{m1J}$ on mTAAR1-D2 function are determined using radioligand binding assays. mTAAR1-D2 binds ligand with very low affinity, more than 60x lower than mTAAR1-B6. Thus, $Taar1^{m1J}$ encodes a receptor with drastically reduced binding affinity relative to the typically functioning mTAAR1-B6. This difference in binding ability could underly the differences in MA intake between the MADR lines as well as their other MA-related phenotypes.

Introduction

As methamphetamine (MA) use and related deaths continue to rise (Han, Compton, et al., 2021; Han, Cotto, et al., 2021), the need for effective treatment options grows more urgent. Pharmacotherapies to aid addiction treatment are attractive options, but have seen limited success. There remain few options for pharmacotherapies for drug addictions, and none for MA addiction specifically. A better understanding of the mechanisms of action of MA, and what biological variables contribute to MA addiction risk can support these research efforts.

The MA drinking lines (MADR) were developed to explore the genetic and biological mechanisms behind MA intake. The MADR lines were selectively bred from an F2 cross of the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains (Shabani et al., 2011; Wheeler et al., 2009). These lines consist of the MA high drinking (MAHDR) and MA low drinking (MALDR) selected lines. MAHDR line mice consistently consume binge-like quantities of MA, and are resistant to the aversive effects of MA (Harkness et al., 2015; Phillips & Shabani, 2015; Shabani et al., 2016; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). In contrast, MALDR line mice consume little-to-no MA and are highly sensitive to the aversive effects of MA (Harkness et al., 2015; Phillips & Shabani, 2015; Shabani, Dobbs, et al., 2012; Shabani, McKinnon, et al., 2012). MALDR line mice also grow markedly hypothermic in response to MA, whereas MAHDR line mice do not (Harkness et al., 2015). They do not, however, display any behavioral or physiological differences in response to cocaine or ethanol (Harkness et al., 2015; Mootz et al., 2020; Wheeler et al., 2009). A quantitative trait locus (QTL) analysis revealed a QTL on mouse chromosome 10 accounting for > 60% of the genetic variance in MA intake between the lines (Belknap et al., 2013). The trace amine-associated receptor 1 gene (*Taar1*) falls within this region. MA is a TAAR1 agonist (Bunzow et al., 2001; Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007), and subsequent studies have identified *Taar1* as the quantitative trait gene (QTG) for the MA intake differences between the MADR lines (Harkness et al., 2015; Phillips et al., 2021; Shi et al., 2016; Stafford et al., 2019).

The receptor encoded by *Taar1* is an intracellular G-protein-coupled receptor (GPCR) expressed in multiple brain regions (Bradaia et al., 2009; Lindemann et al., 2008; Revel et al., 2011; Rutigliano, Accorroni, & Zucchi, 2018; Underhill et al., 2020, 2019; Xie & Miller, 2008; Xie & Miller, 2009b, 2009a; Xie et al., 2007). It is best recognized as a regulator of monoaminergic activity, modifying transporter function and monoamine sequestration in vesicles, reducing monoaminergic neuron signaling (Bradaia et al., 2009; Revel et al., 2011; Rutigliano et al., 2018; Xie & Miller, 2008; Xie & Miller, 2009a; Xie et al., 2007). As the name implies, TAAR1 is activated by endogenous trace amines, such as p-tyramine, β-phenylethylamine, octopamine, and tryptamine (Borowsky et al., 2001; Xie et al., 2007; Zucchi, Chiellini, Scanlan, & Grandy, 2006). Amphetamine and amphetamine-like substances, such as MA, are also TAAR1 agonists (Bunzow et al., 2001; Wolinsky et al., 2007; Zucchi et al., 2006). Because TAAR1 primarily functions in an intracellular environment, MA can only exert its TAAR1-mediated effects once it is inside the cell (Miller, 2011; Underhill et al., 2020, 2019; Xie & Miller, 2007, 2009). MA can gain access to TAAR1 through the serotonin (SERT), norepinephrine (NET), and the dopamine (DAT) transporters (Lindemann et al., 2008; Underhill et al., 2020, 2019; Xie & Miller, 2007, 2008), allowing it to exert its effects in monoaminergic neurons. TAAR1 activation leads to NET and DAT internalization (Underhill et al., 2020, 2019; Xie & Miller, 2008; Xie & Miller, 2009a), and TAAR1 activation can reduce SERT function (Xie & Miller, 2008; Xie et al., 2007).

D2 mice contribute a *Taar1* allele ($Taar1^{m1J}$) with a non-synonymous single-nucleotide polymorphism (SNP) resulting in a proline to threonine residue substitution at amino acid position 77 (P77T), in the second transmembrane domain (Harkness et al., 2015; Keane et al., 2011; Shi et al., 2016; Yalcin et al., 2011). The resulting receptor, mTAAR1-D2, does not produce a cAMP response in the presence of agonists, whereas the TAAR1 expressed by MALDR mice and B6 mice, mTAAR1-B6, does (Harkness et al., 2015; Shi et al., 2016). This led to the conclusion that mTAAR1-D2 may be non-functional (Harkness et al., 2015; Shi et al., 2016). Studies into the intracellular signaling of TAAR1 have revealed that, absent a cAMP

response, the possibility exists that TAAR1 is still exerting an effect on the cell (Underhill et al., 2020, 2019). Activation of TAAR1 engages two intracellular signaling pathways. One pathway is mediated by the G_{13} G-protein α-subunit (Gα), and activates RhoA ultimately internalizing the neuronal glutamate transporter (EAAT3), DAT and NET (Underhill et al., 2020, 2019), and was more recently found to internalize SERT (Underhill & Amara, 2022). The other pathway is mediated by the G_s-subunit. This pathway stimulates cAMP production, leading to PKA-mediated phosphorylating RhoA, halting RhoA activation and presumably the internalization of DAT, EAAT3, and NET (Kang et al., 2007; Underhill et al., 2020, 2019). Whether the same signaling pathways lead to SERT trafficking is not known, but considering the known effects of TAAR1 on SERT function this seems likely (Xie & Miller, 2008; Xie et al., 2007). These effects do not occur sequentially (Underhill et al., 2019). Rather there is a delay between RhoA activation and RhoA phosphorylation, with an initial increase in activated Rho followed by a return to baseline levels (Underhill et al., 2020, 2019, 2014; Wheeler et al., 2015). Because mTAAR1-D2 does not induce cAMP production (Harkness et al., 2015), the G_s-subunit-mediated pathway must not be engaged, likely due to the altered *Taar1^{m1j}* gene product. Whether the G_{13}-subunit-mediated pathway is still recruited is not known. This leaves open the possibility that MA is inducing transporter internalization in monoaminergic neurons in MAHDR mice, and that the internalization is not being halted as it would be in the presence of a typically functioning TAAR1.

The residue substitution resulting from *Taar1^{m1J}* is positioned such that the binding pocket could be rendered incapable of binding ligand (Harkness et al., 2015; Mazna et al., 2008; Reese et al., 2014; Reiersen & Rees, 2001; Sansom & Weinstein, 2000; Schwartz et al., 2006; Van Arnam et al., 2011). If this is the case, neither signaling pathway would be engaged in the presence of agonists, leading to the conclusion that the MA-related differences observed between the MADR lines are likely due to activation of TAAR1 in MALDR mice that is absent in MAHDR mice. If mTAAR1-D2 ligand binding is not impaired, then the MA-related differences

between the MADR lines could be due to differences in TAAR1 recruitment of G_{13}-pathway-mediated effects. Here, we tested the hypothesis that mTAAR1-D2 has markedly impaired capacity to bind ligand. Utilizing a radio-labeled TAAR1 agonist, we probed the binding affinity of mTAAR1-D2 and mTAAR1-B6.

Methods

Drugs and chemicals

The TAAR1 agonists RO5166017 and RO5256390 were purchased from MedChemExpress (New Jersey, USA). [^{3}H]RO5166017 was made by VITRAX (Placentia, CA) from unlabeled RO5166017. Commonly used reagents detailed below were purchased from Sigma Aldrich unless otherwise specified.

Cell culture

Human embryonic kidney cells (HEK-293) were transfected with mTAAR1-D2 (HEK-D2) or mTAAR1-B6 (HEK-B6) (Harkness et al., 2015). Cell culture was performed as previously described (Eshleman et al, 1999, 2013). Stably transfected cells were cultured on 150-mm-diameter tissue culture dishes, in DMEM supplemented with 10% fetal bovine serum (Atlas Biologicals, Fort Collin), 0.1% penicillin/streptomycin, and selected with 600 μg/ml (HEK-D2) or 800 μg/ml (HEK-B6) neomycin (G418). Cell were kept in a humidified 10% CO_2 incubator at 37°C

Receptor binding

Experiments were performed following previously published methods (Eshleman et al., 2020; Toll et al., 1998; Torralva et al., 2020). Both cell lines were grown to confluence before being harvested for membrane preparation. To harvest cells, culture dishes were rinsed with PBS and 10 ml lysis buffer (2mM HEPES, 1mM EDTA) was added. Cells were kept in lysis buffer at 20°C for 10 min. Cells were then scraped from plates and added to 15 mL centrifuge tubes, and centrifuged at 15,500g for 20 minutes at 4°C. Pellets were resuspended in 10mL binding buffer (50 mM Tris, 20 mM HEPES buffer solution, 10 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4

at 4°C) with a Polytron homogenizer, then centrifuged at 15,500g for 20 minutes at 4°C. Pellets were resuspended in 7 mL binding buffer and homogenized.

Receptor binding assays contained the following: 100 µl of membrane preparation (approximately 40 µg protein), 700 µl of binding buffer, 100 µl of [^{3}H]RO5166017, and 100 µl of RO5256390 (nonspecific), RO5166017 (for dilution of [^{3}H]RO5166017 specific activity; see mTAAR1-D2 binding), or an additional 100 µl of binding buffer, for a total volume of 1,000 µl. Incubations were conducted at 4°C for 90 min. Assays were terminated by filtration with a Wallac 96-well harvester through 0.3% polyethylenimine-soaked filters (Perkin Elmer filtermat A). Filters were dried and scintillation fluid added. Radiation remaining on the filters was measured with a Perkin Elmer microbeta plate scintillation counter. To determine mTAAR1-B6 binding, the concentrations of [^{3}H]RO5166017 were 0.31 nM – 3.0 nM, and RO5256390 was used to estimate nonspecific binding. Several iterations of the radioligand binding assay were conducted to determine mTAAR1-D2 binding. In the initial saturation binding experiment, concentrations of [^{3}H]RO5166017 were 0.31 nM – 3.0 nM, and RO5256390 was used to estimate nonspecific binding. This failed to yield calculable K_d and B_{max} values. Subsequent saturation binding experiments were performed by diluting the specific activity of 3 nM [^{3}H]RO5166017 with unlabeled ligand (RO5166017) ranging in concentration from 0.391 to 100 nM or 0.781 nM to 75 nM, or by diluting 50 nM [^{3}H]RO5166017 with unlabeled ligand (RO5166017) at concentrations of 3.906 to 1000 nM. In these studies, RO5256390 was used with the intention of estimating nonspecific binding. However, when [^{3}H]RO5166017 was diluted with higher concentrations of RO5166017, those > 100 nM, RO5166017 outcompeted RO5256390 at binding sites, yielding unusable results. Therefore, the highest concentration of RO5166017 (1000 nM) was used to estimate nonspecific binding. mTAAR1-D2 binding experiments using concentrations of unlabeled RO5166017 of 0.391 nM – 100 nM and 0.781 nM – 75 nM were performed in duplicate. All other experiments, including mTAAR1-B6 binding, were performed in triplicate.

Data analysis

Data were analyzed with GraphPad Prism 7 (San Diego, CA). Specific binding was defined as the difference in binding observed in the presents and absence RO5256390, except when concentrations of RO5166017 ranged from 3.906 to 1000. In these experiments, specific binding was determined by subtracting binding at the highest concentration of RO5166017 (1000 nM) from all other binding values. For all experiments performed in triplicate (all HEK-B6 bindings experiments, HEK-D2 experiments using [^{3}H]RO5166017 concentrations of 0.31 nM – 3.0 nM, and HEK-D2 experiments using RO5166017 concentrations of 3.906 – 1000 nM), the average of the replicates was used to determine K_d and B_{max} values. All other experiments were performed in duplicate and the data were analyzed and are presented for each experiment.

Results

mTAAR1-B6 binding

The concentrations of [^{3}H]RO5166017 were 0.31 nM – 3.0 nM, and RO5256390 was used as the nonspecific. These concentrations yielded a mean K_d value of 0.516 (SEM = 0.240) nM and a B_{max} of 417.969 (SEM = 62.673) fmol/mg protein (Table 2.1 and Figure 2.1a).

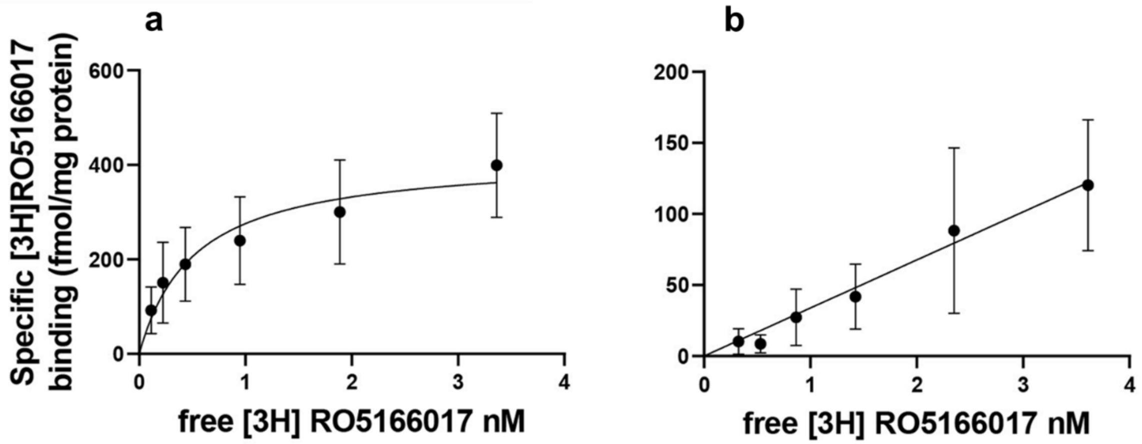

Figure 2.1. Equilibrium saturation binding of [^{3}H]RO5166017 to membranes from (a) HEK-B6 and (b) HEK-D2 cells. Data are means ± SEM of 3 experiments. Note ordinate scale differences for Panels a and b.

mTAAR1-D2 binding

The initial concentrations of [^{3}H]RO5166017 were 0.31 nM – 3.0 nM (Figure 2.1b). These concentrations could not produce consistently calculable K_d and B_{max}, values, indicating we were not saturating the available receptor pool. The specific activity of [^{3}H]RO5166017 was reduced by dilution with 0.391 nM – 100 nM of unlabeled ligand (RO5166017). The two replicates of this experiment did not produce a calculable K_d and B_{max} values. However, we observed a potential inflection point at approximately the 50 nM radioligand concentration (Figure 2.2), suggesting the K_d was near that concentration. We narrowed the concentration of unlabeled ligand (RO5166017) to increase the resolution around the predicted K_d value. Concentrations of unlabeled ligand were narrowed to 0.781 nM – 75 nM. The two replicates of this experiment produced K_d values of 84.82 nM and 58.97 nM, and B_{max} values of 5997 nM and 8504 nM (Figure 2.3).

These K_d values were near the highest concentration used. Rather than perform additional replicate experiments using these concentrations, we decided to increase the minimum and maximum concentrations to achieve a range that encompassed the K_d value and upward of 10x the K_d. value. Thus, the concentrations of unlabeled ligand were then increased to 3.906 nM – 1000 nM. RO5256390 was initially used to estimate nonspecific binding, however the high concentrations of RO5166017 outcompeted RO5256390 at mTAAR1, yielding unusable results. Therefore, the highest concentration of unlabeled RO5166017 was used to calculate nonspecific binding (see *Data analysis*). Thus, the final concentrations of unlabeled RO5166017 used to dilute [^{3}H]RO5166017 were 3.906 nM – 500 nM. Estimated K_d and B_{max} values and SEMs for each are shown in Table 2.1 and equilibrium binding curves are shown in Figure 2.4.

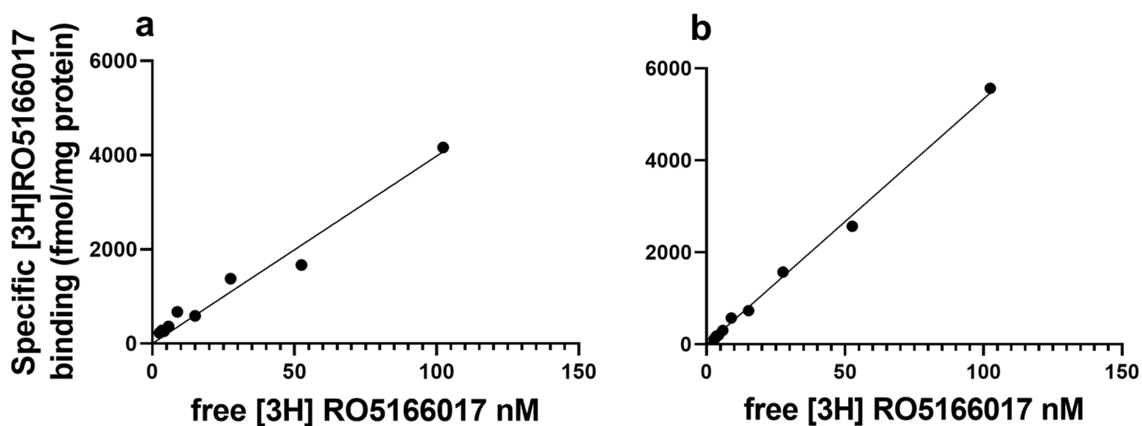

Figure 2.2. Equilibrium saturation binding of [^{3}H]RO5166017 to membranes from HEK-D2 cells. Data in (a) and (b) are from 2 experiments. Potential inflections points can be observed at approximately the 50 nM concentration. Saturation binding was performed by diluting the specific activity of [^{3}H]RO5166017 with unlabeled RO5166017 up to 100 nM as described in *Materials and Methods*.

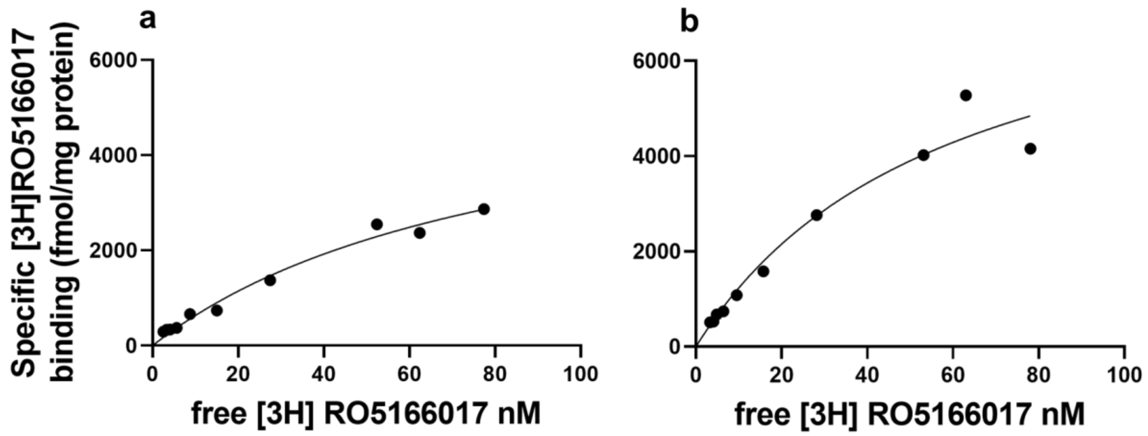

Figure 2.3. Equilibrium saturation binding of [^{3}H]RO5166017 to membranes from HEK-D2 cells. Data in (a) and (b) are from 2 experiments. Saturation binding was performed by diluting the specific activity of [^{3}H]RO5166017 with unlabeled RO5166017 up to 75 nM as described in *Materials and Methods*.

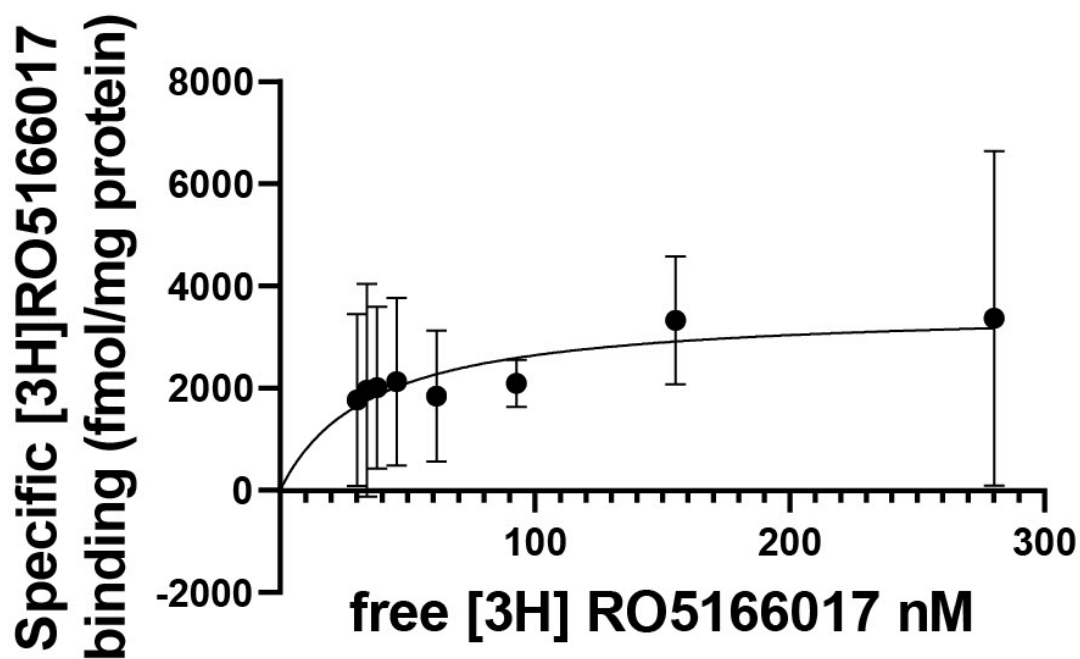

Figure 2.4. Equilibrium saturation binding of [^{3}H]RO5166017 to membranes from HEK-D2 cells. Data are means ± SEM of 3 experiments. Saturation binding was performed by diluting the specific activity of [^{3}H]RO5166017 with unlabeled RO5166017 up to 1,000 nM as described in *Materials and Methods*.

Table 2.1. [^{3}H]RO5166017 equilibrium binding to mTAAR1-B6 and mTAAR1-D2 expressing HEK cells. Assays were conducted as described in the text. Values left blank were ambiguous

Cell Line	Concentrations [^{3}H]RO5166017 (nM)	Nonspecific	K_d (SEM)	B_{max} (SEM)
Triplicate (mean values)				
HEK-B6	0.31-3.0	RO5256390	0.516 (0.240)	417.969 (62.673)
HEK-D2	0.31-3.0	RO5256390		
HEK-D2	50.0-500.0	RO5166017	35.075 (33.141)	3573.807 (1182.215)
Duplicate (individual experiments)				
HEK-D2	0.781-75	RO5256390	84.82	5997
HEK-D2	0.781-75	RO5256390	58.97	8504
HEK-D2	0.391-100	RO5256390		
HEK-D2	0.391-100	RO5256390		

Discussion

Here, we present the first evidence that a naturally occurring SNP of *Taar1* results in a receptor with dramatically decreased ability to bind ligand. The change in binding due to *Taar1*^{m1J} is not unprecedented given the location of the residue substitution. P77T falls close to residue V75, predicted to be a binding pocket stabilizing residue (Reese et al., 2014). The substitution of a proline has particular significance given proline provides molecular dynamics involved with, and often necessary for, proper receptor function (Mazna et al., 2008; Reiersen & Rees, 2001; Sansom & Weinstein, 2000; Schwartz et al., 2006; Van Arnam et al., 2011). No existing data could conclusively determine P77T would alter ligand binding though, necessitating the experiments presented here. Because *Taar1* is a QTG for the MA intake differences between the MADR lines (Harkness et al., 2015; Phillips et al., 2021; Shi et al., 2016; Stafford et al., 2019), the loss of receptor function due to impaired MA binding ability is likely responsible for much of the genetic variance in MA intake between the MADR lines.

Confirming these functional differences permits more complete hypotheses about the role of TAAR1 in MA intake. For many purposes MAHDR line mice can be considered functional *Taar1* knockout (KO) mice. It is tempting to conclude that the MA-related differences observed between the MADR lines are due to the acute effects of MA at TAAR1, and thus the lack of TAAR1 activation in MAHDR line mice. However, several alternative explanations must be considered. The reduced binding at mTAAR1-D2 does not preclude constitutive activity, which may be different in mTAAR1-D2 vs mTAAR1-B6 expressing mice. Such constitutive activity differences between the respective B6 and D2 mTAAR1 variants has not been directly explored. However, research into the MADR lines and TAAR1 in general can help generate predictions.

Research using *Taar1*$^{-/-}$ mice demonstrates that TAAR1 is tonically active and impacts neuronal activity (Bradaia et al., 2009; Lindemann et al., 2008; Wolinsky et al., 2007; Leo et al., 2014). It is important to note that all of these studies utilized *Taar1*$^{-/-}$ mice derived from a B6 background, or a mix of B6 and some other inbred strain, and their wild-type (WT) littermates.

Every mouse strain tested, other than D2 mice from Jackson Laboratory, express a functional TAAR1 (Reed et al., 2017; Shi et al., 2016). It can thus be assumed that any differences between *Taar1*$^{-/-}$ mice and their WT littermates are due to the genetic deletion of TAAR1 and not some differences of innate receptor function due to genetic backgrounds. Relative to WT littermates, *Taar1*$^{-/-}$ mice have elevated extracellular dopamine in the NAcc (Leo et al., 2014). Midbrain dopamine neurons of *Taar1*$^{-/-}$ mice exhibit elevated spontaneous firing compared to WT mice-derived neurons (Bradaia et al., 2009; Lindemann et al., 2008). These differences could be due to either tonic activation of TAAR1 by endogenous ligand, constitutive activity of TAAR1, or both. Tonic activity was confirmed when application of a TAAR1 antagonist in tissue derived from WT mice increased spontaneous firing to rates comparable to those found in *Taar1*$^{-/-}$ mouse tissue (Bradaia et al., 2009). Evidence for constitutive activity was found as well. In HEK cells expressing human TAAR1, a TAAR1 antagonist reduced cAMP production below baseline in the absence of other ligands (Bradaia et al., 2009), indicating the antagonist was actually functioning as a reverse agonist, and thus reduced constitutive, ligand-independent TAAR1 activity. Since the data presented here show that mTAAR1-D2 does not bind ligand unless agonist concentrations are far above those required to bind mTAAR1-B6, and above the concentrations found for endogenous trace amines (Pei et al., 2016), we can assume that any effects resulting from tonic activation of TAAR1 are absent in mTAAR1-D2-expressing mice and *Taar1*$^{-/-}$ mice. If the spontaneous firing rates of WT mouse midbrain DA neurons and NAcc DA levels are due predominantly to tonic activation of TAAR1 by endogenous ligand, one would expect mTAAR1-D2-expressing mice to display phenotypes similar to *Taar1*$^{-/-}$ mice. If ligand-independent constitutive activity plays a large role we cannot accurately predict how mTAAR1-D2 neurons might behave.

It is worth discussing some conflicting evidence surrounding the role of TAAR1 in mediating DA release. In one study, in vivo microdialysis recordings of *Taar1*$^{-/-}$ and WT mice found no difference in baselines striatal dopamine, serotonin, or noradrenaline, nor any

difference in DAT expression (Lindemann et al., 2008). Leo et al. (2014) found that *Taar1*$^{-/-}$ mice had differences in extracellular dopamine in the NAcc, but not the dorsal striatum (Leo et al., 2014), providing clear regional differences in TAAR1-mediated effects. In another study using striatal tissue from *Taar1*$^{-/-}$ and WT mice, radioligand binding assays found no differences in DAT expression, nor were there differences in baseline or MA-associated DAT function (Miner, Phillips, & Janowsky, 2019). This is in direct contrast to other studies demonstrating TAAR1 activation decreases DAT function. In studies using cultured cells expressing human or monkey TAAR1 and DAT, TAAR1 agonists decreased DAT function (Underhill et al., 2019; Xie & Miller, 2007, 2008; Xie & Miller, 2009a), and earlier studies comparing *Taar1*$^{-/-}$ and WT striatal tissue found TAAR1 agonists decrease DAT function (Xie & Miller, 2007, 2008; Xie & Miller, 2009a). First, all the evidence collected from cultured cells agrees that TAAR1 mediates DAT function. TAAR1 agonists invariably reduce DAT function in cultured HEK cells expressing TAAR1 and DAT (Underhill et al., 2019; Xie & Miller, 2007, 2008; Xie & Miller, 2009a; Xie et al., 2007). Where the data disagree is when studying tissue collected from animals. When considering the methods of the respective papers, there may not actually be a conflict. As mentioned, Leo et al. (2014) found that *Taar1*$^{-/-}$ mice had differences in extracellular regional differences in TAAR1-mediated effects on DA levels (Leo et al., 2014). Most other studies investigating TAAR1 function in tissue extracted the midbrain or whole striatum, and different dissection methods may yield different proportions of NAcc and dorsal striatum tissue. Taken together disagreements between studies could be a result of unaccounted for region-specific effects of TAAR1.

mTAAR1-D2 could also trigger compensatory adaptations, and these adaptations might be the underlying source of MA intake differences and other MA-related behaviors. Since TAAR1 antagonists applied to *Taar1*$^{+}$ tissue replicate the neuronal activity and dopamine release of *Taar1*$^{-/-}$ mice, it is not likely compensatory mechanisms form within the cell in which mTAAR1-D2 is expressed. This does not prohibit more distal adaptations though.

TAAR1 has become a prospective pharmacological target for a number of diseases (Cao, Li, Wu, & Li, 2021; Pei et al., 2016; Revel et al., 2011; Schwartz et al., 2018; Wolinsky et al., 2007; Wu & Li, 2021). Considering TAAR1 activation mediates aversive effects of MA (Harkness et al., 2015; Phillips et al., 2021; Phillips & Shabani, 2015; Shabani et al., 2016; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009), it could be a promising target for MA addiction treatment. The results presented here suggest TAAR1 drugs may not be effective in some individuals, or may require higher doses depending. SNPs that affect human TAAR1 function have already been documented (Shi et al., 2016),. There are broader implications as well. The existence of a naturally occurring point mutation that both enhances addiction risk, in this case MA addiction risk, and eliminates a promising drug target for that disease is troubling. This highlights the importance of continued research into the downstream mechanisms of TAAR1 activation, as these may offer viable treatment targets in the absence of a fully functional TAAR1. It also stresses the value of revisiting reports that conclude a potential pharmacotherapy is ineffective, either in preclinical or clinical trials, as previously unknown genetic variations may alter drug efficacy leading to erroneous conclusions about drug efficacy.

It is unlikely mTAAR1-D2 is capable of binding MA better than the artificial ligand used here. A competition binding assay, in which the ability of MA to block radioligand binding is measured, would have been ideal for this. Seeing as the affinity of mTAAR1-D2 for our radioligand was so low, we had no way of performing this. However, RO5166017 is a highly selective and potent TAAR1 agonist (Cichero, Espinoza, Gainetdinov, Brasili, & Fossa, 2013; Leo et al., 2014; Revel et al., 2011). If it had bound to mTAAR1-D2 with greater affinity there would be reason to believe MA might bind and activate TAAR1 to some extent, but with the observed levels of RO5166017 binding it is not probable any TAAR1 agonists can bind to mTAAR1-D2 with greater efficacy than reported here. To date, only one purported binding pocket has been modeled (Lindemann et al., 2005; Reese et al., 2014).

The B_{max} value for mTAAR1-D2 was nearly 10x greater than that for mTAAR1-B6. This aligns with previous western blot data measuring TAAR1 levels in transfected HEK-293 cells (Harkness et al., 2015), suggesting greater mTAAR1-D2 expression. We do not have a good explanation for this, but it does indicate TAAR1 possesses some form of self-regulatory expression that requires ligand binding. This could be through modulation of TAAR1 transcription, or degradation of receptors. Because mTAAR1-D2 binds ligand so poorly, this overexpression of the mTAAR1-D2 likely does not contribute to any effects associated with TAAR1 activation, such as any of the MA-specific phenotypes of the MADR lines.

Future studies should more thoroughly examine the effects of mTAAR1-D2 expression on cell function and neural signaling. Transporter trafficking has not been examined in the MADR lines. Since TAAR1 is a known regulator of monoamine transporter trafficking (Underhill et al., 2022, 2020, 2019; Xie & Miller, 2007, 2008; Xie & Miller, 2009b), it is worthwhile to examine DAT, SERT, and NET in the MADR lines, both at baseline and after MA exposure. Using CRISPR-Cas9, MAHDR line mice have been generated that possess the B6 *Taar1* allele, thus expressing mTAAR1-B6. These could be used to determine specific mTAAR1-D2 molecular and neurochemical effects. Constitutive effects of mTAAR1-D2 could be assessed with resonance energy transfer sensors, much in the same way other studies have done to measure TAAR1 signaling pathways (Underhill et al., 2020, 2019).

The results of TAAR1 activation consist of at least two components, initial RhoA activation and subsequent monoamine transporter and EAAT3 internalization, followed by eventual PKA activation which halts that process (Underhill et al., 2020, 2019). As TAAR1 activation contributes to aversive MA effects (Harkness et al., 2015; Phillips et al., 2021; Phillips & Shabani, 2015; Shabani et al., 2016; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009), it is pertinent to determine which signaling pathways mediate aversive effects. One way to approach this would be to target a downstream effector of RhoA activation using a Rho-kinase inhibitor. Rho-kinases are directly associated with amphetamine-induced DAT function (Wheeler

et al., 2015). These are safe to use in vivo (Inan & Büyükafşar, 2008), and could be used in MALDR mice, or other mTAAR1-B6 expressing mouse lines to establish the role of RhoA activation in aversive effects of MA.

Here we present evidence that a SNP in mouse *Taar1* greatly reduces ligand binding to TAAR1. This impaired ability to effectively bind ligand is likely the cause of the effects of *Taar1* genotype on MA intake between the MADR lines. Constitutive activity and compensatory adjustments cannot be ruled out. For instance, since the absence of TAAR1 increases spontaneous DA neuron firing, neurons may develop mechanisms to mitigate this increased signaling. The profound behavioral and physiological effects of MA in mice with functional TAAR1 that are absent in mice with nonfunctional or at least altered function TAAR1 argues for a central role of TAAR1 activation in the MA intake differences between the MADR lines. This highlights the need to include considerations of genetic variation in best practices for preclinical and clinical studies of pharmacotherapies for addiction.

Chapter 3:

Differential genetic risk for methamphetamine intake confers differential sensitivity to the temperature-altering effects of other addictive drugs

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Abstract

Mice selectively bred for high methamphetamine (MA) drinking (MAHDR), compared to mice bred for low MA drinking (MALDR), exhibit greater sensitivity to MA reward and insensitivity to aversive and hypothermic effects of MA. Previous work identified the trace amine-associated receptor 1 gene (*Taar1*) as a quantitative trait gene for MA intake that also impacts thermal response to MA. All MAHDR mice are homozygous for the mutant $Taar1^{m1J}$ allele, whereas all MALDR mice possess at least one copy of the reference $Taar1^{+}$ allele. To determine if their differential sensitivity to MA-induced hypothermia extends to drugs of similar and different classes, we examined sensitivity to the hypothermic effect of the stimulant cocaine, the amphetamine-like substance 3,4-methylenedioxymethamphetamine (MDMA), and the opioids morphine and buprenorphine in these lines. The lines did not differ in thermal response to cocaine, only MALDR mice exhibited a hypothermic response to MDMA, and buprenorphine induced hypothermia in both lines but not to the same magnitude as morphine. MAHDR mice were more sensitive to the hypothermic effect of morphine than MALDR mice. We speculated that the μ-opioid receptor gene (*Oprm1*) impacts morphine response, and genotyped the mice tested for morphine-induced hypothermia. We report genetic linkage between *Taar1* and *Oprm1*; MAHDR mice more often inherit the $Oprm1^{D2}$ allele and MALDR mice more often inherit the $Oprm1^{B6}$ allele. Data from a family of recombinant inbred mouse strains support the influence of *Oprm1* genotype, but not *Taar1* genotype, on thermal response to morphine. These results nominate *Oprm1* as a genetic risk factor for morphine-induced hypothermia that does not extend to buprenorphine, and provide additional evidence for a connection between drug preference and drug thermal response.

Introduction

Chronic methamphetamine (MA) use is linked to numerous deleterious health effects and an increased mortality rate (Kuo et al., 2011; Stenbacka, Leifman, & Romelsjö, 2010). Genetic variation impacts risk for MA addiction (Aoyama et al., 2006; Ehlers, Gizer, Gilder, & Wilhelmsen, 2011) and rodent research supports the contribution of genetic variation to MA sensitivity, which could impact use. For example, in mice, genetic variation impacts locomotor (Kim et al., 2018; Parker, Cheng, Sokoloff, & Palmer, 2012) and thermal responses (Harkness et al., 2015), as well as sensitivity to rewarding and aversive effects of MA (Clough, Hutchinson, Hudson, & Dubocovich, 2014; Harkness et al., 2015; Kim et al., 2018; Wheeler et al., 2009). Our lab created the selectively bred MA drinking (MADR) mouse lines, comprised of MA high drinking (MAHDR) and MA low drinking (MALDR) lines, to investigate genetic influences on risk for MA intake and genetic relationships between MA sensitivity traits and MA intake. The MADR lines do not differ in the amounts of several novel tastants voluntarily consumed, including quinine, potassium chloride, and saccharin (Shabani, Dobbs, et al., 2012; Wheeler et al., 2009), and they consume similar amounts of MA on the first day it is offered (Eastwood et al., 2014; Shkelzen Shabani, Dobbs, et al., 2012). This suggests the difference in MA consumption between the lines on subsequent days is not due to differential sensitivity to the taste of MA, but rather to differences in sensitivity to effects of MA experienced on the initial day of consumption. Further, sensitivities to reinforcing, rewarding, and aversive MA effects are genetically correlated with MA intake in the MADR lines, suggesting that some of the genes impacted by selective breeding have pleiotropic influences on these traits. Accordingly, MAHDR mice operant self-administer MA and display MA-conditioned reward, but not MA-conditioned aversion, whereas MALDR mice are phenotypically opposite in their MA-related responses (Shabani et al., 2011; Shabani, Dobbs, et al., 2012; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). Notably, following an MA injection MALDR mice become hypothermic, whereas MAHDR mice do not, a result we have consistently obtained across replicated sets of the MADR

lines (Harkness et al., 2015). Although MA is typically characterized for its hyperthermic effects, at normothermic ambient temperatures MA can induce hypothermia (Harkness et al., 2015), which may be protective against MA-induced neurotoxicity (Miller & O'Callaghan, 1994). Hypothermia can also increase the period during which negative associations are conditioned (Misanin et al., 2002). Thus, hypothermia experienced after MA consumption may enhance the association of MA with subjective aversive effects.

The MADR lines represent an animal model of differential genetic risk for MA use (Phillips & Shabani, 2015). To identify the genes that may confer high vs. low risk, we performed a quantitative trait locus (QTL) analysis and identified a region on mouse chromosome 10 accounting for 60% of the genetic variance in MA intake between the MADR lines (Belknap et al., 2013). The trace amine-associated receptor 1 gene (*Taar1*), at 23.9 Mb on chromosome 10, was identified as a major contributor (Harkness et al., 2015; Stafford et al., 2019). We discovered a spontaneous mutation within the coding region of *Taar1* in one of the founder strains of the MADR lines, the DBA/2J (D2) inbred strain, and found that this mutant allele ($Taar1^{m1J}$) codes for a nonfunctional form of the receptor (TAAR1) (Harkness et al., 2015). The other founder strain, C57BL/6J (B6), contributes the reference allele ($Taar1^{+}$), which codes for a functional TAAR1 and is present in all 28 other mouse strains that have been examined (Reed et al., 2017; Shi et al., 2016). TAAR1 is an intracellular g protein-coupled receptor (GPCR) activated by endogenous trace amines, monoamines and amphetamines (Bunzow et al., 2001; Miller, 2005; Xie & Miller, 2009b), and modulates monoamine transmission and reuptake (Revel et al., 2011; Xie & Miller, 2008; Xie & Miller, 2009b). In the 5 replicate sets of MADR lines we have produced, selective breeding for MA intake has resulted in homozygosity for $Taar1^{m1J}$ in all MAHDR mice, with the majority of MALDR mice homozygous for $Taar1^{+}$ and none possessing more than one copy of the mutant allele (Harkness et al., 2015; Reed et al., 2017). We also considered the µ-opioid receptor gene (*Oprm1*), at 6.75 Mb on chromosome 10, for its role in the difference between the MADR lines in MA intake and found that *Oprm1* is not directly

associated with risk for MA intake (Eastwood et al., 2018). Rather, it serves as a “hub” for regulation by the top-ranked transcription factor differential gene expression network for MA intake risk (Belknap et al., 2013).

In addition to impacting MA intake, TAAR1 functionality impacts the hypothermic response to MA. *Taar1* knockout mice do not become hypothermic following MA treatment, whereas their wild-type (WT) littermates do (Harkness et al., 2015; Miner, Elmore, Baumann, Phillips, & Janowsky, 2017). Likewise, *Taar1*$^{+/+}$ mice from recombinant inbred (RI) mouse strains derived from the F2 cross of the B6 and D2 progenitors (the BXD RI strains) become hypothermic, whereas *Taar1*$^{m1J/m1J}$ BXD RI mice do not (Reed et al., 2017; Stafford et al., 2019). Further, D2 mice from The Jackson Laboratory, which are homozygous for the mutant *Taar1*^{m1J} allele, are insensitive to MA-induced hypothermia, whereas D2 mice from 3 other vendors, which are homozygous for the reference *Taar1*$^{+}$ allele, become hypothermic after MA treatment (DenHerder et al., 2017). Furthermore, *Taar1*$^{m1J/m1J}$ D2 mice consume significantly more MA than do *Taar1*$^{+/+}$ D2 or B6 mice (Eastwood & Phillips, 2014b; Reed et al., 2017).

The MADR lines do not differ in thermal response to ethanol (Harkness et al., 2015). The effects of other addictive drugs on body temperature have not been examined in these mice, and may provide information about pleiotropic gene actions across drugs. Here, we investigated the effects of the psychostimulant cocaine, the amphetamine-like substance 3,4-methylenedioxy methamphetamine (MDMA), and the opioid morphine, all of which can induce hypothermia (Belknap et al., 1998; Di Cara et al., 2011; Ishizuka, Rockhold, Hoskins, & Ho, 1990; Miner, O’Callaghan, Phillips, & Janowsky, 2017). The MADR lines differ in sensitivity to MA-conditioned, but not cocaine-conditioned reward and aversion (Gubner et al., 2013), suggesting distinct mechanisms contribute to these effects of MA vs. cocaine. We predicted that the MADR lines would display comparable thermal responses to cocaine, but since MDMA and MA are both amphetamine-like drugs and TAAR1 agonists (Berry, Gainetdinov, Hoener, & Shahid, 2017; Simmler, Buchy, Chaboz, Hoener, & Liechti, 2016), MDMA would induce hypothermia

only in MALDR mice. As MAHDR mice consume less of the full OPRM1 agonist morphine than MALDR mice (Eastwood & Phillips, 2014a) and a greater hypothermic response to MA corresponds with lower MA intake (Harkness et al., 2015), we predicted that MAHDR mice would exhibit greater morphine-induced hypothermia than MALDR mice. We examined associations of *Taar1* and *Oprm1* genotype with morphine-induced temperature change, and the results of this study led us to explore the potential independent and interactive influences of *Taar1* and *Oprm1* genotypes on morphine-induced thermal effects. Finally, we examined the effect of the partial OPRM1 agonist buprenorphine on body temperature. We wished to determine whether the MADR lines would exhibit a thermal response difference to this partial OPRM1 agonist, similar to the difference observed for the full agonist, morphine. Further, because buprenorphine, when administered prior to initial MA access, reduced MA intake in MAHDR mice (Eastwood & Phillips, 2014a), we were particularly interested in whether buprenorphine induces hypothermia in these mice, an effect that could serve as an aversive associative stimulus, subsequently reducing MA intake. Because buprenorphine and morphine are both opioids that act on OPRM1, albeit in different capacities, and the MADR lines differ in morphine intake, we hypothesized buprenorphine would induce hypothermia in both MADR lines, however MAHDR line mice would be more sensitive to this hypothermic effect.

Methods

Animals

Prior to experimentation, all mice were group-housed in polycarbonate shoebox cages (28.5 × 17.5 × 12 cm) with wire tops and Bed-O'Cobs bedding (The Andersons, Maumee, OH, USA). Mice had free access to rodent food (Purina 5001 or 5LOD PicoLab Rodent Diet; Animal Specialties, Woodburn, OR) and were maintained on a 12:12 hour light:dark cycle with lights on at 0600 hour. Colony room temperature was 21 ± 1 °C. 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 Veterans Affairs Portland Health Care System (VAPORHCS) Institutional Animal Care and Use Committee

Participating in the MADR experiments were 305 male and 308 female MAHDR and MALDR mice, ages 58-102 days. Numbers for each experiment are given below. All mice were experimentally naïve at the time of testing. Details of selective breeding have been previously published (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). Briefly, each replicate set of the MADR lines was selectively bred from a founding population of B6D2F2 mice. The choice of breeders for each selection generation was based on voluntary consumption of a 40 mg/l MA solution consumed in a 2-bottle choice drinking procedure, during which they had access to water and 20 mg/l MA for 4 days and then water and 40 mg/l MA for 4 days. Thus, the mice that consumed the highest average amounts of MA were chosen to establish and perpetuate the MAHDR line, whereas the lowest consumers established and perpetuated the MALDR line. Results across replicate for response to selection have been reproducible (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). For Experiment 1, mice were from selection generation 5 (S5) of the fourth replicate set of the MADR lines (157 mice) and from S1-S3 of the fifth replicate set (178 mice). Replicate 5 was under development at the time of these studies, and only early selection generation mice were available. We did not see this as a problem, because the greatest divergence in MA intake between the lines occurs in S1 (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009), due to the major impact of *Taar1* on MA intake and the nearly complete fixation of the *Taar1*^{m1J} allele within the first generation of selection (Reed et al., 2017). MADR line differences have been highly reproducible (Harkness et al., 2015; Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). For Experiment 3, all 278 mice were from S5 of the fifth replicate set of MADR lines.

Participating in Experiment 2 were 120 female and 120 male BXD RI mice, ages 59-84 days. All mice were experimentally naïve at the time of testing. Breeding pairs of BXD RI strains

were obtained from Dr. Robert Williams (University of Tennessee Health Science Center, Memphis, TN), and established within the VAPORHCS. Specific strains were chosen based on their combined *Taar1* and *Oprm1* genotypes and breeding potential. In total, there were 14 BXD RI strains that had four genotypes: *Taar1*$^{+/+}$/*Oprm1*$^{B6/B6}$ (n=16 BXD184, n=26 BXD154, n=12 BXD196, and n=6 BXD218); *Taar1*$^{m1J/m1J}$/*Oprm1*$^{B6/B6}$ (n=27 BXD161, n=8 BXD199, and n=25 BXD205); *Taar1*$^{+/+}$/*Oprm1*$^{D2/D2}$ (n=22 BXD113, n=12 BXD171, and n=26 BXD194); *Taar1*$^{m1J/m1J}$/*Oprm1*$^{D2/D2}$ (n=17 BXD178, n=18 BXD186, n=5 BXD210, and n=20 BXD216), evenly distributed by strain across the dose groups.

Drug-induced core body temperature changes

Core body temperature was assessed using established procedures (Harkness et al., 2015; Reed et al., 2017). All experiments were performed during the light phase, between 0900 and 1300 hour. Mice were weighed, isolated in acrylic cubicles to prevent huddling-associated body temperature changes, and left undisturbed for 1 hour to allow acclimation to the testing environment, also maintained at 21 ± 1 °C. Following acclimation, baseline temperature was taken at time 0 (T0), using a 5 mm rectal probe attached to a Thermalert TH-8 digital thermometer (Sensortek, Clifton, NJ). Mice were then immediately treated with vehicle or the appropriate drug dose, placed back into their holding cubicles, and removed to obtain temperatures at T15, T30, T60, T90, T120, and T150 min post-injection.

Drugs

Cocaine hydrochloride was purchased from Sigma (St. Louis, MO). MDMA, buprenorphine sulfate, and morphine sulfate were obtained from the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA). All drugs were dissolved in sterile physiological saline (0.9% NaCl, Baxter Healthcare Corporation, Deerfield, IL) and injected intraperitoneally in a 10 ml/kg volume.

Experiment 1

Thermal responses to the cocaine, MDMA, and morphine were assessed in a single study, so that a common saline control group could be used, allowing fewer animals to be included. The study was completed in 4 equal passes in S5 mice of the fourth replicate and S1 mice of the fifth replicate of the MADR lines (140 mice total; 5/sex/line/drug-specific dose or saline). Doses of cocaine were 15 and 30 mg/kg, MDMA doses were 2.5 and 5 mg/kg, and morphine doses were 15 and 30 mg/kg. These doses were chosen based on a literature review demonstrating behavioral and thermal effects (Eastwood & Phillips, 2014b; Ishizuka et al., 1990; Miner, O'Callaghan, et al., 2017). Following the final temperature recording, mice were euthanized and tail samples taken for genotyping.

For morphine, initial analyses identified sex and dose effects. Because the group size per sex and dose was small for the initial morphine study, there were concerns about reliability of effects. Therefore, 6 additional passes of 14-42 mice were added, increasing the total sample size to 20-23 mice per sex, line, and saline or morphine dose (95 additional replicate 4 S5 mice and 100 additional replicate 5 S2 and S3 mice). One mouse died during the experiment and its data were excluded.

Experiment 2

BXD RI mice were used to investigate the respective impacts of *Oprm1* and *Taar1* genotype on thermal response to morphine. Procedures and morphine doses were as in Experiment 1. A total of 240 mice were tested for a final group size of 10 per sex, *Oprm1/Taar1* genotype, and drug dose.

Experiment 3

Buprenorphine-induced hypothermia was investigated in MADR mice (10/sex/line/dose). Buprenorphine doses were 1, 2, 4, 8, 16, and 32 mg/kg. The range of doses were chosen based on a literature review demonstrating behavioral effects at these doses without inducing toxicity (Eastwood & Phillips, 2014a; Glovak, Angel, O'Brien, Baghdoyan, & Lydic, 2022; Guarnieri et al., 2012; Lizasoain, Leza, & Lorenzo, 1991) and a pilot study indicating a range of thermal

effects at these doses. We intentionally chose a range of doses that encompassed the doses that had reduced MA intake in MAHDR line mice in a previous study (Eastwood & Phillips, 2014a).

Genotyping

Genomic DNA from the morphine-treated and buprenorphine-treated MADR mice was extracted using QuickExtract DNA extraction solution (Epicenter, Madison, WI). *Oprm1* was amplified using a Hotstart polymerase kit (Qiagen, Valecia, CA) with sequence-specific primers surrounding the region of interest (forward 5'-ggttatgcctctctggattag-3', reverse 5'-tccatcgcttacatcttacca-3'). To determine *Oprm1* genotype, amplified polymerase chain reaction (PCR) products were run on an agarose gel that was scanned on a Bio-Rad Gel Doc™ XR+ Imaging System to determine band intensity. *Taar1* was amplified (forward 5'-ctttctgctgggctgtctga-3', reverse 5'-caacagcgctcaacagttctc-3') and genotype was determined using an rtPCR assay developed in our lab (Harkness et al., 2015; Reed et al., 2017) and based on standard Taqman procedures (Shen, Abdullah, & Wang, 2009).

Statistical Analysis

Data were analyzed using Statistica 13 Academic software (TIBCO Software Inc., Palo Alto, CA, USA). Body temperature data were analyzed by repeated measures ANOVA, with time as the repeated measure and line, sex, drug treatment, *Oprm1* genotype, and *Taar1* genotype as possible independent variables. Effects were considered significant at $p < 0.05$. Significant interactions were examined with simple main effects analyses, and Neuman–Keuls *post hoc* mean comparisons were performed when appropriate. Correlations between *Taar1* and *Oprm1* genotype and temperature change were determined with Pearson's r. Observed versus expected *Taar1* and *Oprm1* genotype frequencies were assessed with the chi-square test. Outliers were considered body temperatures greater than or less than 2.5 standard deviations from the mean and excluded from analysis. Three mice from Experiment 3 were excluded due to this.

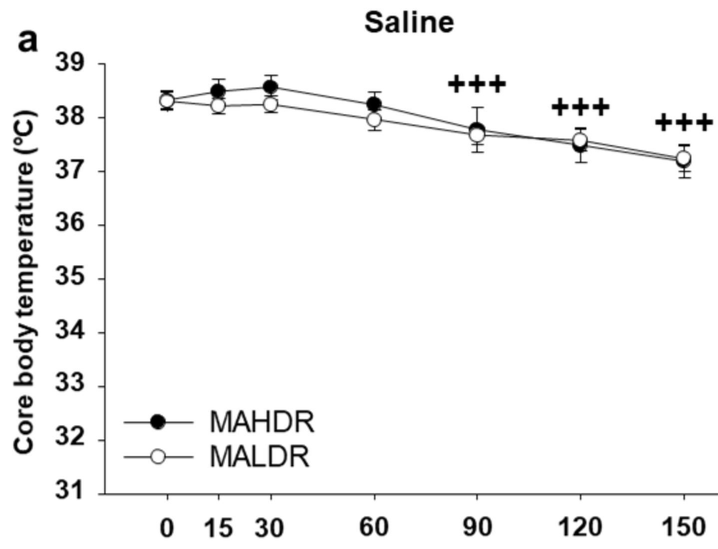
Results

Experiment 1: Thermal responses to multiple drugs in MADR mice

Cocaine

Data are presented in Figure 3.1. In the initial multifactor ANOVA, there were significant time x sex ($F_{6, 288} = 2.1$, $p < 0.05$) and time x dose interactions ($F_{12, 288} = 21.0$, $p < 0.001$, but no significant effects involving line. When the time x sex interaction was examined, there was a significant effect of time within each sex (ps < 0.001), but no significant sex difference at any time point was found. When the time x dose interaction was examined, we found that temperatures were dependent on time for each dose, including saline (ps < 0.05; Figure 3.1a-c). Mice treated with saline had significantly lower body temperatures compared to T0 at T90-T150. Mice treated with the 15 mg/kg cocaine dose had significantly lower body temperatures at T150 compared to T0, but their temperatures did not differ from T0 at any other time point. The 30 mg/kg cocaine-treated mice were hypothermic at T15, but by T30, their mean temperature was not significantly different from T0.

For the effect of dose at each time, there were no significant differences at T0 or T30, but at T15, the 30 mg/kg cocaine group had a significantly lower mean body temperature than the saline or 15 mg/kg cocaine groups (ps < 0.001). At T60-T150, the 30 mg/kg cocaine group had significantly higher mean body temperatures, compared to the saline group (ps < 0.001 - 0.05) and the mean temperature of this group was also higher than that of the 15 mg/kg cocaine dose group at T150 ($p < 0.05$). These differences are not indicated by symbols in Figure 3.1, due to representation of the effects of each dose in separate panels for clarity.


a
Saline
Core body temperature (ºC)
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MAHDR
MALDR
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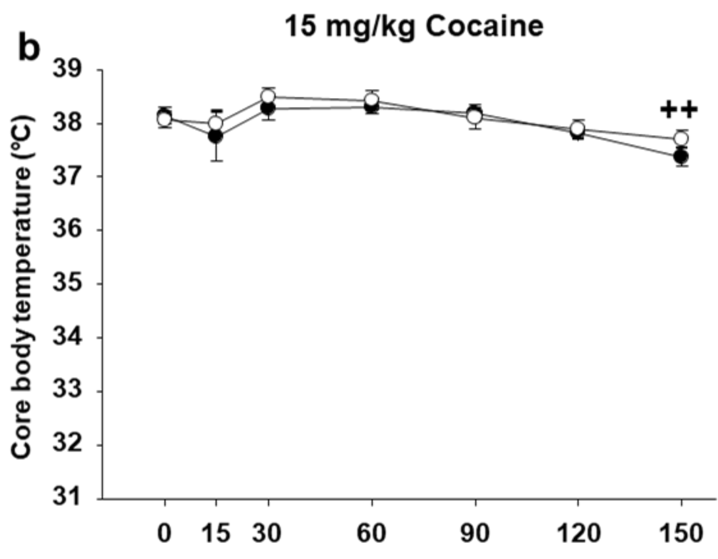
b
15 mg/kg Cocaine
Core body temperature (ºC)
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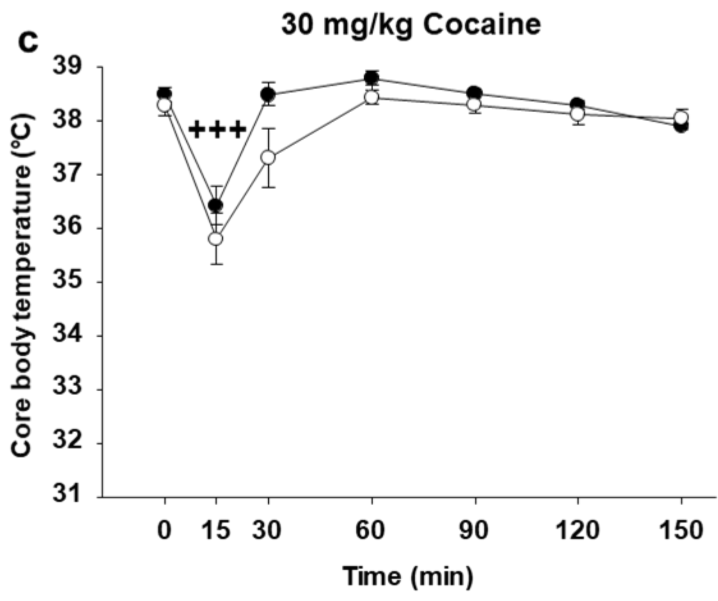
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Core body temperature (ºC)
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Time (min)

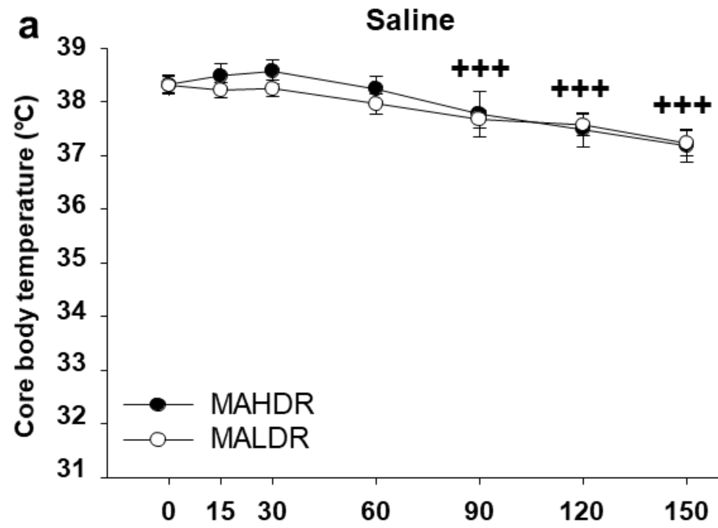
Figure 3.1. Mice bred for high and low methamphetamine (MA) intake exhibit similar sensitivity to the hypothermic effects of cocaine. There were no significant effects of sex involving line or cocaine dose, therefore the data are presented for the sexes combined. Shown are the effects of (a) saline, (b) 15 mg/kg cocaine, and (c) 30 mg/kg cocaine on core body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. ++$p < 0.01$, and +++$p < 0.001$ for temperature change from T0 at the indicated time point for the main effect of time. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking

MDMA

Data are presented in Figure 3.2. The initial multifactor ANOVA identified significant time x line x dose ($F_{12, 288} = 2.0$, $p < 0.05$) and time x sex ($F_{6, 288} = 4.5$, $p < 0.001$) interactions. Females had higher T0 and T150 temperatures than males (ps < 0.05), but the sexes did not differ at any other time point. There was a significant effect of time within each sex (ps < 0.001). Next, the significant 3-way interaction was examined by repeated measures time x line ANOVAs for each dose. For the saline group (Figure 3.2a), only the effect of time was significant ($F_{6, 108} = 20.6$, $p < 0.001$), with decreases in mean temperature at T90-T150, regardless of line. For the 2.5 mg/kg MDMA group (Figure 3.2b), there was a significant time x line interaction ($F_{6, 108} = 4.7$, $p < 0.001$), with a significant effect of time in both lines (ps < 0.001 - 0.05). Compared to T0, the mean temperature of MAHDR mice was significantly higher at T30 and significantly lower at T120 and T150 (Figure 3.2b); however, these differences in temperature did not exceed 0.5 °C. No significant changes in body temperature from T0 were detected for the MALDR line by post-hoc analysis. However, following treatment with the 2.5 mg/kg dose of MDMA, the mean temperature of MALDR mice was significantly lower than that of MAHDR mice at T15 and T60 (Figure 3.2b). For the 5 mg/kg MDMA group (Figure 3.2c), there was a significant time x line interaction ($F_{6, 108} = 6.3$, $p < 0.001$), with a significant effect of time only in the MALDR line ($p < 0.001$). Compared to T0, the mean temperature of the MALDR line was significantly lower at T15 and T30. The maximal decrease in temperature was approximately 2°C, occurring at T15. In addition, the mean temperature of MALDR mice was significantly lower than that of MAHDR mice at T15 and T30 after treatment with this MDMA dose.

We next examined the effect of dose at each time point for each line. At T0, there was no effect of dose. At T15, temperature was dependent on dose for both lines (ps < 0.001 - 0.05). There were no differences between the saline and 2.5 mg/kg MDMA groups for either line, but mean body temperatures of the 5 mg/kg-treated MALDR and MAHDR groups were lower than those of the other 2 treatment groups (all ps < 0.001 - 0.05), with more profound effects in the

MALDR line. At T30, temperatures were dependent on dose only in the MALDR line ($p < 0.001$), with significantly lower mean temperature for the 5 mg/kg group, compared to the other 2 groups ($ps < 0.01 – 0.05$). There were no significant dose-dependent effects at any other time point. These differences are not indicated by symbols in Figure 3.2, due to representation of the effects of each dose in separate panels for clarity.


a
Saline
Core body temperature (°C)
MAHDR
MALDR
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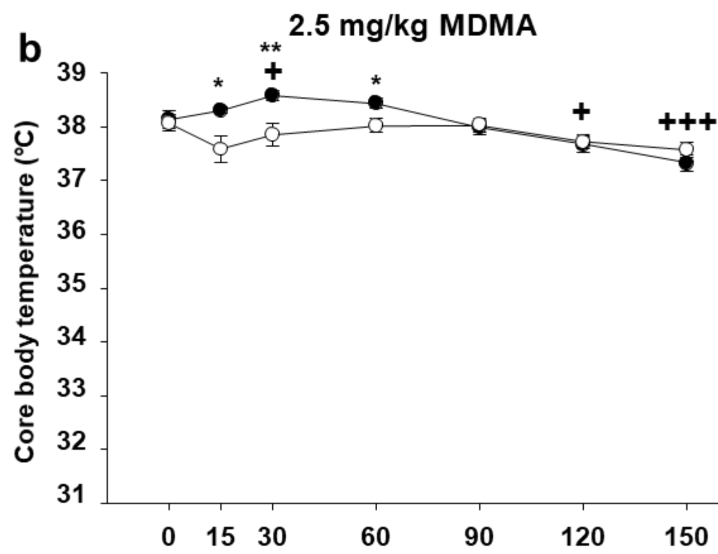
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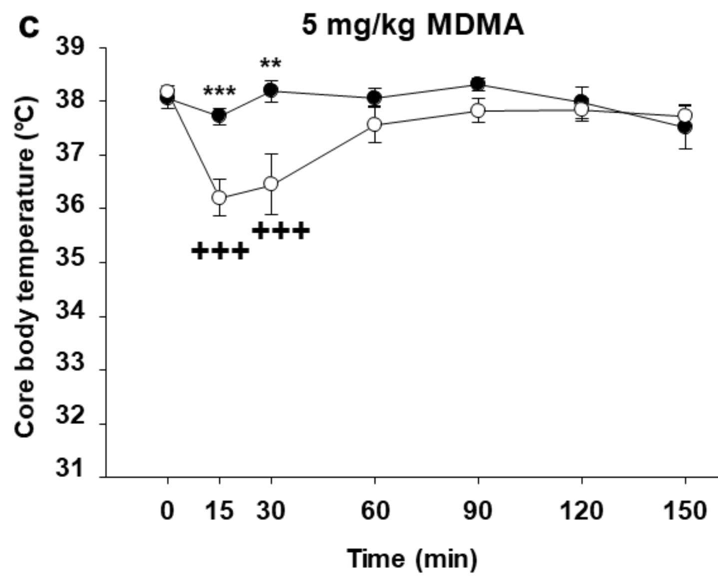
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Time (min)

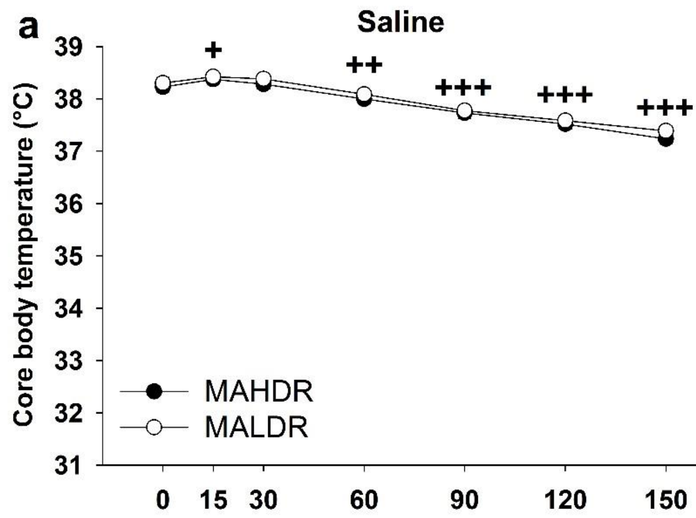
Figure 3.2. Mice bred for low methamphetamine (MA) intake are more sensitive to hypothermic effects of MDMA on core body temperature than mice bred for high methamphetamine intake. There were no significant effects of sex involving line or dose, so the data are presented for the sexes combined. Shown are the effects of (a) saline, (b) 2.5 mg/kg MDMA, and (c) 5 mg/kg MDMA on body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. +$p < 0.05$ and +++$p < 0.001$ for temperature change from T0 at the indicated time point for (a) the main effect of time, (b) the MAHDR line, and (c) the MALDR line; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for the difference between the lines at the indicated time point. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking; MDMA: 3,4-methylenedioxymethamphetamine

Morphine

Data are presented in Figure 3.3. In the initial multifactor ANOVA, there were significant time x line x dose ($F_{12, 1452} = 4.5$, $p < 0.001$) and time x sex x dose interactions ($F_{12, 1452} = 2.0$, $p < 0.05$). First, data were examined for time and sex effects within each dose. Temperatures were dependent on time for both sexes at all doses (all ps < 0.001). A significant time x sex interaction was found for the saline ($F_{6, 498} = 4.5$, $p < 0.001$) and 30 mg/kg morphine ($F_{6, 486} = 2.98$, $p < 0.01$) groups. Compared to females, male saline-treated mice had lower temperatures of less than 0.5°C, and only at T150 ($p < 0.01$). Males in the 30 mg/kg morphine group also had lower temperatures, but only at T0 ($p < 0.001$).

Because sex did influence line-dependent responses to morphine, data for the sexes were combined and a significant time x line x dose interaction was confirmed ($F_{12, 1488} = 4.3$, $p < 0.0001$). For the saline group, there was a significant effect of time ($F_{6, 498} = 100.0$, $p < 0.001$), due largely to a progressive reduction in body temperature (Figure 3.3a). For each morphine dose, there was a significant time x line interaction ($F_{6, 504} = 8.78$, $p < 0.001$ and $F_{6, 486} = 6.78$, $p < 0.001$ for 15 and 30 mg/kg, respectively). For the 15 mg/kg dose, both the MAHDR and MALDR lines exhibited time-dependent hypothermia (ps < 0.001) and the mean temperature of MAHDR mice was significantly lower than that of MALDR mice at every time point (Figure 3.3b). The thermal response followed a biphasic pattern, characterized by a rapid decrease in temperature after injection followed by an increase in temperature. The mean difference between the 2 lines of mice was 0.4°C at baseline, compared to a maximal difference of 2.2°C at T60. For the 30 mg/kg dose, both lines displayed hypothermia (ps < 0.001), that persisted across the entire measurement period (Figure 3.3c). MAHDR mice had significantly lower mean temperatures than MALDR mice, at every time point, except T0. When time and dose effects were examined, there was a significant time x dose interaction for each line ($F_{12, 744} = 45.1$, $p < 0.001$ and $F_{12,744} = 39.6$, ps < 0.001 for MAHDR and MALDR, respectively). For each line, the effect of dose was significant at every time point, except T0 (ps < 0.001). Temperature changed

significantly across time for saline and each morphine dose ($ps < 0.001$), with a linear decrease in the saline group, and biphasic responses in the morphine groups.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Saline
Core body temperature (°C)
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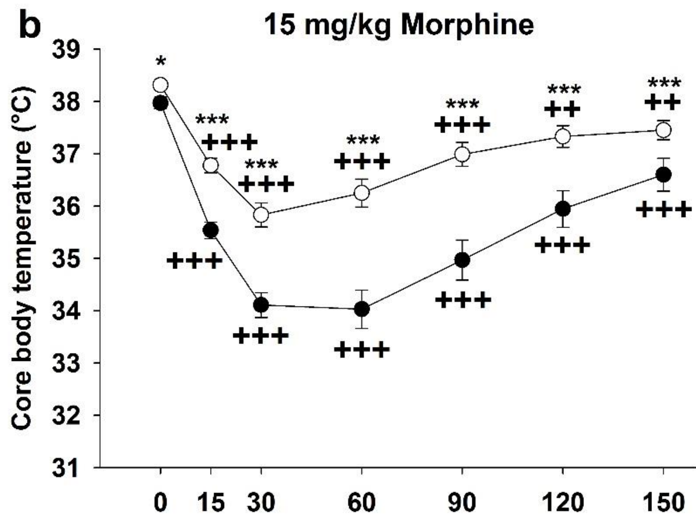
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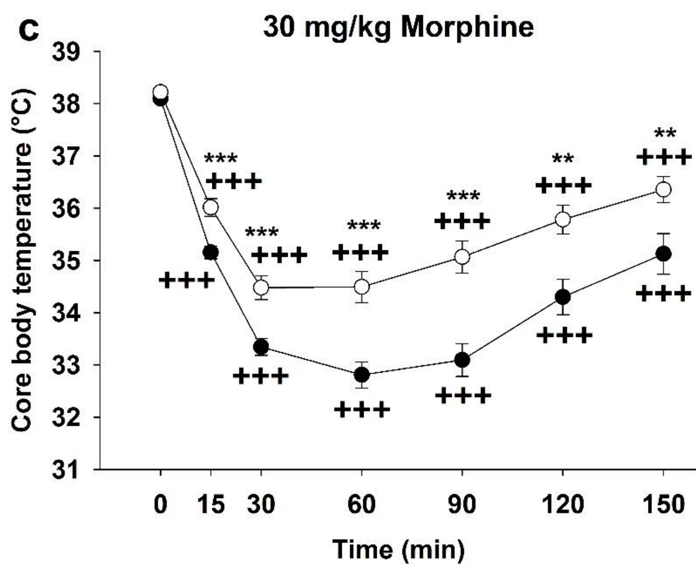
c
30 mg/kg Morphine
Core body temperature (°C)
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0 15 30 60 90 120 150
Time (min)

Figure 3.3. Mice bred for high methamphetamine (MA) intake are more sensitive to the hypothermic effects of morphine than mice bred for low methamphetamine intake. Because sex did not play a role in line-dependent responses to morphine, the data are presented for the sexes combined. Shown are the effects of (a) saline, (b) 15 mg/kg morphine, and (C) 30 mg/kg morphine on core body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. +$p < 0.05$, ++$p < 0.01$, and +++$p < 0.001$ for temperature change from T0 at the indicated time point for (a) the main effect of time; (b and c) the MAHDR or MALDR line; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for the difference between the lines at the indicated time point. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking

Morphine: genotyping results and correlations in MADR mice

The genotyping results for *Taar1* and *Oprm1* in the MADR lines are summarized in Figure 3.4. MAHDR mice were predominantly *Oprm1*$^{D2/D2}$ and *Oprm1*$^{D2/B6}$, whereas MALDR mice were predominantly *Oprm1*$^{B6/B6}$. For *Taar1*, MAHDR mice were almost entirely *Taar*$^{m1J/m1J}$; a single animal from S1 replicate 5 was *Taar1*$^{+/m1J}$. MALDR mice were almost entirely *Taar1*$^{+/+}$ or *Taar1*$^{+/m1J}$; a single animal from S1 replicate 5 was *Taar1*$^{m1J/m1J}$. If there had been no impact of selective breeding on the frequency of *Oprm1* or *Taar1*, the expected ratio of the different possible genotypes for each gene (*Oprm1*$^{B6/B6}$: *Oprm1*$^{B6/D2}$: *Oprm1*$^{D2/D2}$ and *Taar1*$^{+/+}$: *Taar1*$^{+/m1J}$: *Taar*$^{m1J/m1J}$) would be 1 : 2 : 1, within each line or 31.75 : 63.5 : 31.75 for each of the 127 MAHDR and MALDR mice tested in this study. Chi-square tests indicated that the observed ratios differed significantly from the expected ratios for both the MAHDR (chi-squared = 106 and 373 for *Oprm1* and *Taar1*, respectively, ps < 0.001) and MALDR (chi-squared = 284.4 and 197.9 for *Oprm1* and *Taar1*, respectively, ps < 0.001) lines.

Correlations were separately calculated between thermal response to morphine and *Oprm1* or *Taar1* genotype. There were insufficient mice of every possible combined *Oprm1* and *Taar1* genotype to assess potential correlations with allele combinations. In fact, there was a significant correlation between progenitor source of *Oprm1* and *Taar1* ($r = 0.80$, $p < 0.01$), so that *Taar1* genotype predicted *Oprm1* genotype 64% of the time, indicating linkage disequilibrium for these 2 genes. Because the hypothermic response to morphine was greatest at T60, a change value was calculated for each mouse as temperature at T60 minus temperature at T0 (T60-T0), and used to calculate correlations with genotype (D2 or B6 allele, or *m1J* or + allele, homozygous or heterozygous) for each dose (Table 3.1). For the saline group, the correlations with *Oprm1* genotype ($r = 0.06$, $p > 0.05$) and *Taar1* genotype ($r = 0.00$, $p < 0.05$) were not significant. For 15 mg/kg morphine, there were significant correlations with both *Oprm1* ($r = 0.50$, $p < 0.01$) and *Taar1* ($r = 0.48$, $p < 0.01$) genotype. Similarly, for 30 mg/kg

morphine, there were significant correlations with *Oprm1* ($r = 0.44$, $p < 0.01$) and *Taar1* ($r = 0.43$, $p < 0.01$) genotype.

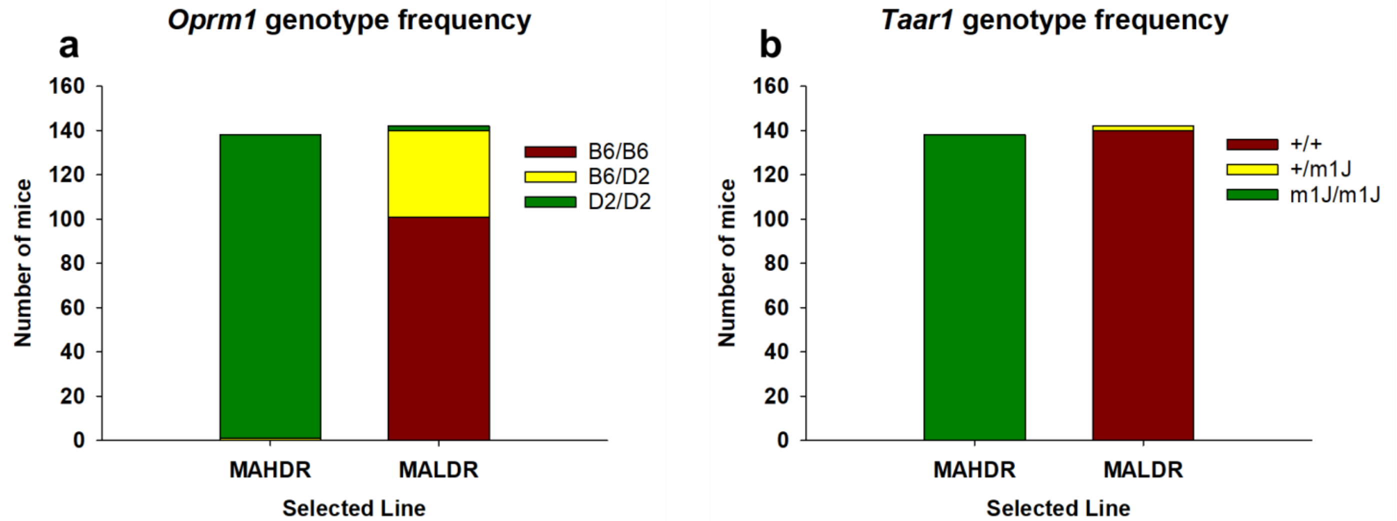

Figure 3.4. (a) *Oprm1* and (b) *Taar1* genotype frequencies for methamphetamine high and low drinking mice tested in the study of morphine thermal effects. +: reference *Taar1* allele; B6: C57BL/6J; D2: DBA/2J; *m1J*: mutant *Taar1* allele found only in D2 mice; MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking; *Oprm1*: mu-opioid receptor gene; *Taar1*: trace amine-associated receptor 1 gene

Experiment 2: Thermal response to morphine in BXD RI mice

Due to linkage disequilibrium, we could not study the individual contributions of *Oprm1* and *Taar1* genotype in the MADR lines. Therefore, BXD RI mice were tested in which the linkage is disrupted. Results are summarized in Figure 3.5 for *Oprm1* genotype (Figure 3.5a-c) and *Taar1* genotype (Figure 3.5d-f). The analysis for which the results are presented did not include strain as a factor, rather the BXD RI mice were considered as a single population, since they were all derived from the D2 and B6 progenitor strains. This afforded a large population size of related individuals for examination of the genetic effects (see same approach in our published paper[20]). There were significant *Oprm1* x *Taar1* x sex ($F_{1, 216} = 4.8$, $p < 0.05$), time x sex ($F_{5, 1080} = 4.5$, $p < 0.001$), time x *Oprm1* x *Taar1* ($F_{5, 1080} = 5.8$, $p < 0.0001$), and time x *Oprm1* x dose ($F_{10, 1080} = 11.9$, $p < 0.0001$) interactions. However, there were no interactions of sex and morphine dose nor of *Taar1* genotype and morphine dose, indicating that neither sex nor *Taar1* genotype (Figure 3.5d-f) impacted the response to morphine. Thus, we examined the significant time x *Oprm1* x dose interaction, because it is relevant to the question of whether genotype impacts response to morphine (Figure 3.5a-c).

Within the saline group (Figure 3.5a), there was a significant time x *Oprm1* interaction ($F_{5, 390} = 2.4$, $p < 0.05$). Temperature decreased across time for both genotypes (ps < 0.01), differing from T0 at each time point, but the genotypes did not significantly differ from each other at any time point. For 15 mg/kg morphine (Figure 3.5b), the time x *Oprm1* interaction was significant ($F_{5, 390} = 15.24$, $p < 0.001$). For both genotypes, the 15 mg/kg morphine dose had time-dependent biphasic effects (ps < 0.001) and mice of both genotypes displayed significant hypothermia at all post-injection measurement times. *Oprm1*$^{D2/D2}$ mice had significantly lower mean temperatures than *Oprm1*$^{B6/B6}$ mice at T30-T120 (Figure 3.5b). For 30 mg/kg morphine (Figure 3.3c), the time x *Oprm1* interaction was significant ($F_{5, 390} = 23.51$, $p < 0.001$). Both genotypes exhibited time-dependent, biphasic hypothermia (ps < 0.001). *Oprm1*$^{D2/D2}$ mice had significantly lower temperatures than *Oprm1*$^{B6/B6}$ mice at T30-T150 (Figure 3.5c).

When correlations were calculated between temperature change (T60-T0) and *Taar1* or *Oprm1* genotype for each morphine dose group (Table 3.2), there were no significant correlations for the saline group or with *Taar1* genotype, regardless of morphine dose. However, the correlation with *Oprm1* genotype was significant for both the 15 mg/kg ($r = 0.49$, $p < 0.01$) and the 30 mg/kg ($r = 0.63$, $p < 0.01$) morphine doses.

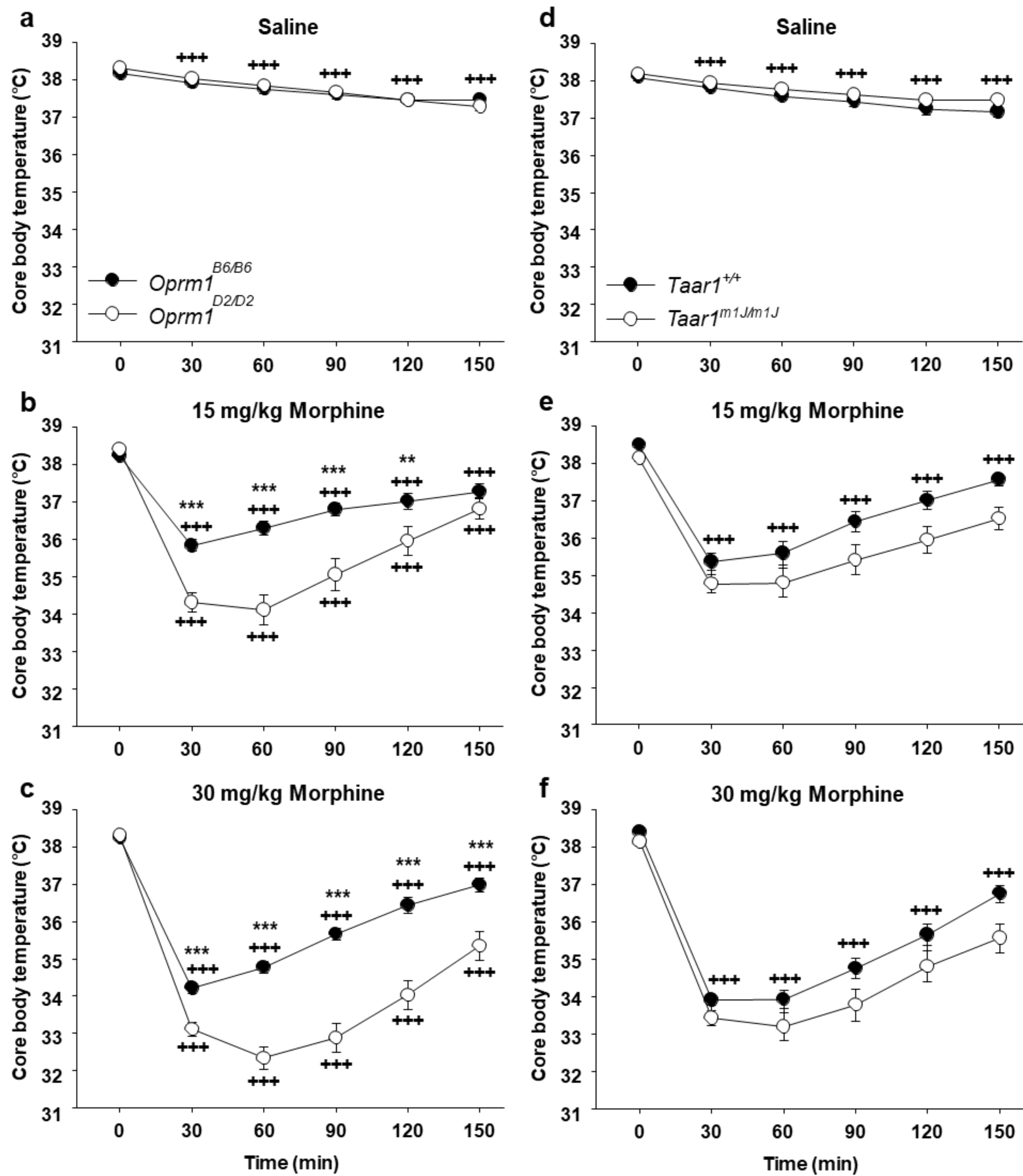

Figure 3.5. Mice possessing the *Oprm1$^{D2/D2}$* genotype are more sensitive to the hypothermic effects of morphine than *Oprm1$^{B6/B6}$* mice. There were no interactions of sex and morphine dose, therefore data are presented for the sexes combined. Shown are the effects of saline, 15 mg/kg morphine, and 30 mg/kg morphine on core body temperature across time in minutes (min) for

BXD RI mice with (a-c) *Oprm1*$^{B6/B6}$ and *Orpm1*$^{D2/D2}$ genotypes and (d-f) *Taar1*$^{+/+}$ and *Taar1*$^{m1J/m1J}$ genotypes. +++$p < 0.001$ for temperature change from T0 at the indicated time point for the main effect of time (a,d-f) or for the *Oprm1*$^{B6/B6}$ or *Oprm1*$^{D2/D2}$ genotype (b,c); **$p < 0.01$, ***$p < 0.001$ for differences between genotypes at the indicated time point. +: reference *Taar1* allele; B6: C57BL/6J; D2: DBA/2J; *m1J*: mutant *Taar1* allele found only in D2 mice; *Oprm1*: mu-opioid receptor gene; *Taar1*: trace amine-associated receptor 1 gene

Experiment 3: Thermal response to buprenorphine

Data for buprenorphine-induced hypothermia are presented in Figures 3.6, 3.7, and 3.8. An initial repeated measures ANOVA including line, time, sex, and buprenorphine dose found a significant line x dose x sex x time interaction ($F_{36, 1500} = 2.0$, $p < 0.05$). Effects within each sex were next examined. There was a significant line x dose x time interaction within both males ($F_{36, 738} = 2.5$, $p < 0.001$) and females ($F_{36, 762} = 1.7$, $p < 0.01$). Data for each sex were next examined for effects of line and time within each dose. Results for males are shown in Figure 3.6a-g. For the saline group (Figure 3.6a), there was no significant line x time interaction, but there was a significant main effect of time ($F_{6, 108} = 22.9$, $p < 0.001$) due to a progressive time-dependent reduction in body temperature, similar to that observed in previous experiments. Also in males, there was a significant effect of time for the 2 ($F_{6, 108} = 7.17$, $p < 0.001$), 8 ($F_{6, 102} = 5.58$, $p < 0.001$), and 16 ($F_{6, 102} = 14.2$, $p < 0.001$) mg/kg MA doses; however there were no significant line effects at these doses. For all of these doses, mice exhibited significant hypothermia at T15 (ps < 0.05; Figure 3.6c,e,f), the magnitude of which increased with dose, and mice returned to baseline temperatures by T60. There were significant time x line interactions for the 1 ($F_{6, 102} = 2.67$, $p < 0.05$), 4 ($F_{6, 108} = 2.54$, $p < 0.05$), and 32 ($F_{6, 102} = 5.8$, $p < 0.001$) mg/kg doses. For the 1 and 4 mg/kg doses, male MALDR mice developed significant hypothermia by T15 (ps < 0.01; Figure 3.6b,d) whereas male MAHDR mice did not. On the other hand, both lines developed significant hypothermia at T15 for the 32 mg/kg dose (ps < 0.05; Figure 3.6g), but MAHDR mice had a significantly greater reduction in body temperature than MALDR mice.

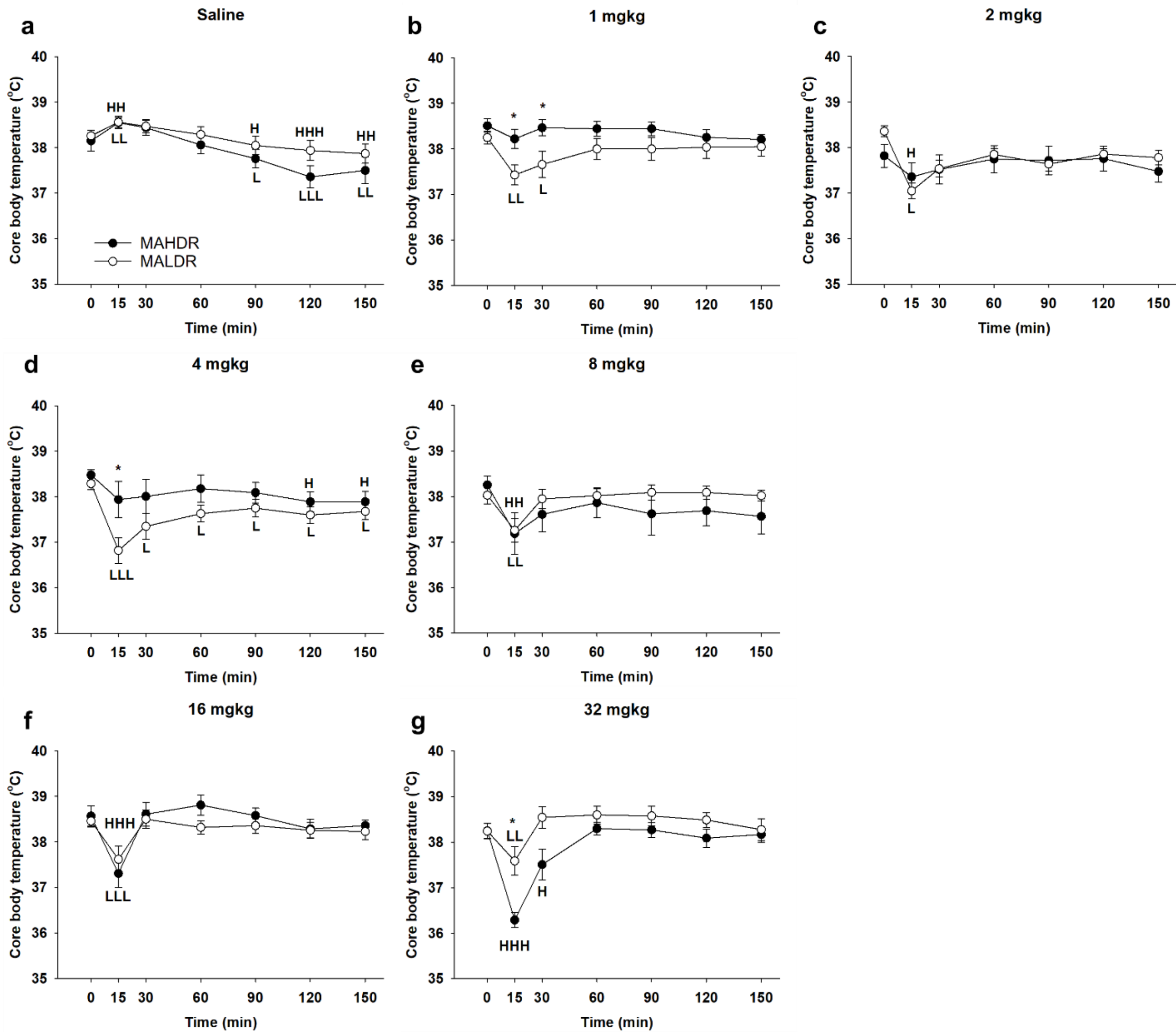

Figure 3.6. Male mice bred for low methamphetamine (MA) intake are more sensitive to the hypothermic effects of certain doses of buprenorphine than mice bred for high MA intake. There were significant effects of sex so the data were analyzed separated by sex. Shown are the effects of (a) saline, (b) 1 mg/kg, (c) 2 mg/kg, (d) 4 mg/kg, (e) 8 mg/kg, (f) 16 mg/kg, (g) 32 mg/kg buprenorphine on body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. **L**$p < 0.05$ and **LL**$p < 0.01$, **LLL**$p < 0.001$ for temperature change from T0 at the indicated time point for the MALDR line, **H**$p < 0.05$ and **HH**$p < 0.01$, **HHH**$p < 0.001$; *$p < 0.05$ for the difference between the lines at the indicated time point. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking

When data for females were examined for effects of line and time within each dose, there were significant main effects of time ($F_{6, 108} = 29.4$, $p < 0.001$) and line ($F_{1, 108} = 16.5$, $p < 0.001$), but no interaction, in the saline group. Saline-treated female MAHDR line mice maintained a lower body temperature than MALDR mice (Figure 3.7a). With regard to the effect of time, saline-treated mice got progressively colder as the experiment progressed (Figure 3.7a). For MA-treated mice, there was a significant main effect of time in females for the 4 ($F_{6, 108} = 14.27$, $p < 0.001$), 8 ($F_{6, 114} = 9.3$, $p < 0.001$), and 16 ($F_{6, 108} = 11.05$, $p < 0.01$) mg/kg doses, but no significant effect of line or line x time interaction. At these doses, mice grew significantly hypothermic at T15 (ps < 0.01; Figure 3.7d,e,f) and then recovered. Finally, there was a significant line x time interaction for the 1 ($F_{6, 108} = 3.2$, $p < 0.01$), 2 ($F_{6, 102} = 2.33$, $p < 0.05$), and 32 ($F_{6, 114} = 2.7$, $p < 0.05$) mg/kg doses. At these doses, female MAHDR mice developed significant hypothermia at T15 (ps < 0.01; Figure 3.7b,c,g) and remained hypothermic for at least 30 minutes, whereas female MALDR mice developed hypothermia only after treatment with the 1 and 32 mg/kg doses (ps < 0.05; Figures 3.7b,g), and returned to base line by T30.

Because the main hypothesis of interest is that buprenorphine induces hypothermia in MAHDR mice and serves as an aversive associative stimulus, the effects of time, sex, and dose were examined within the MAHDR alone. There were no significant interactions of sex and dose; so, to maximize detection of dose-dependent effects, further analyses were performed collapsed on sex. For all buprenorphine doses, MAHDR mice developed hypothermia by T15 (ps < 0.05) and returned to baseline by T90 (Figures 3.8b-g), with greater reductions in body temperature at the 16 and 32 mg/kg doses (Figures 3.8f-g).

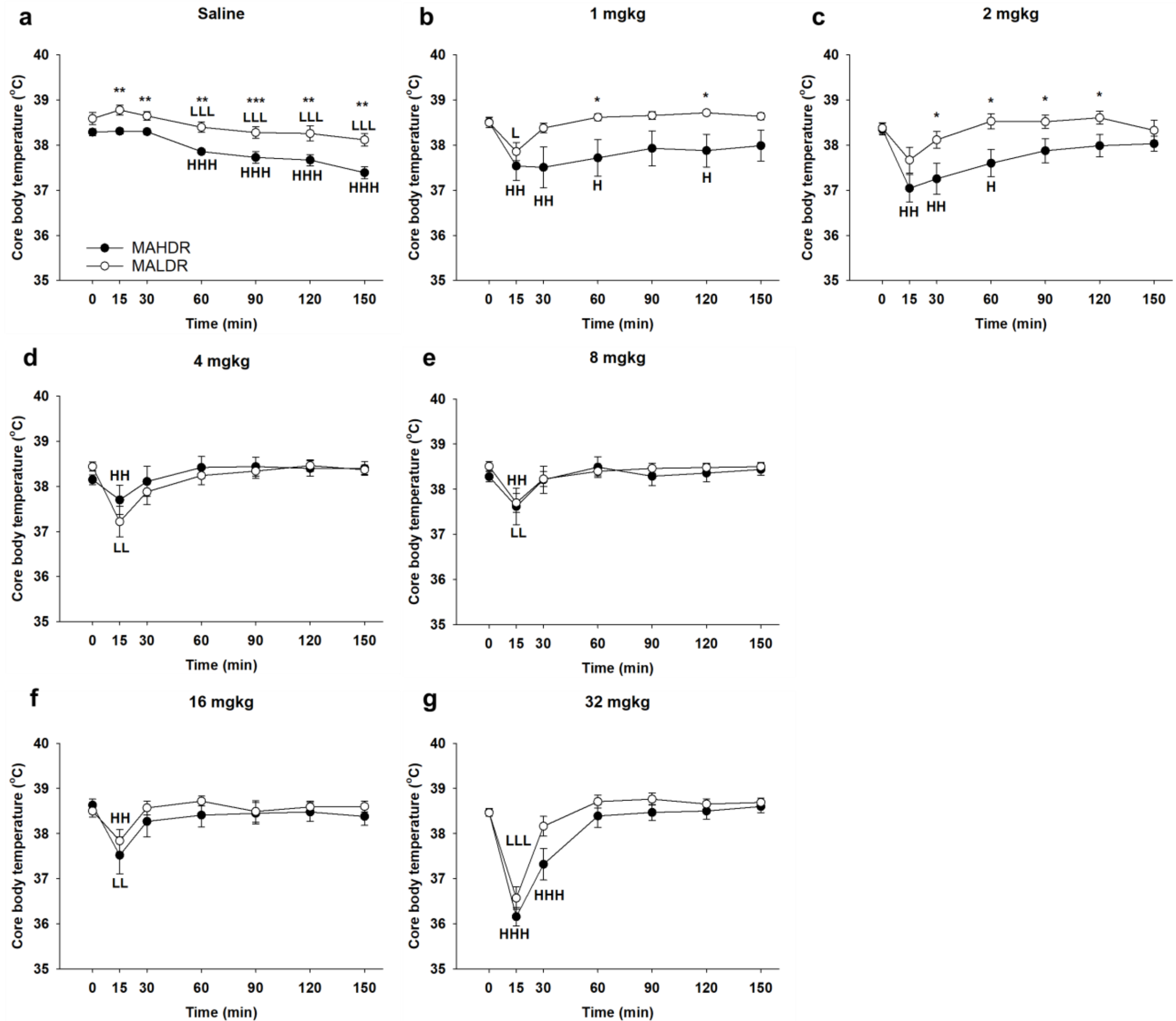

Figure 3.7. Female mice bred for high methamphetamine (MA) intake are more sensitive to the hypothermic effects of certain doses of buprenorphine than mice bred for low MA intake. There were significant effects of sex so the data were analyzed separated by sex. Shown are the effects of (a) saline, (b) 1 mg/kg, (c) 2 mg/kg, (d) 4 mg/kg, (e) 8 mg/kg, (f) 16 mg/kg, (g) 32 mg/kg buprenorphine on body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. **L**p < 0.05 and **LL**p < 0.01, **LLL**p < 0.001 for temperature change from T0 at the indicated time point for the MALDR line, **H**p < 0.05 and **HH**p < 0.01, **HHH**p < 0.001; *p < 0.05, **p < 0.01, and ***p < 0.001 for the difference between the lines at the

indicated time point. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking

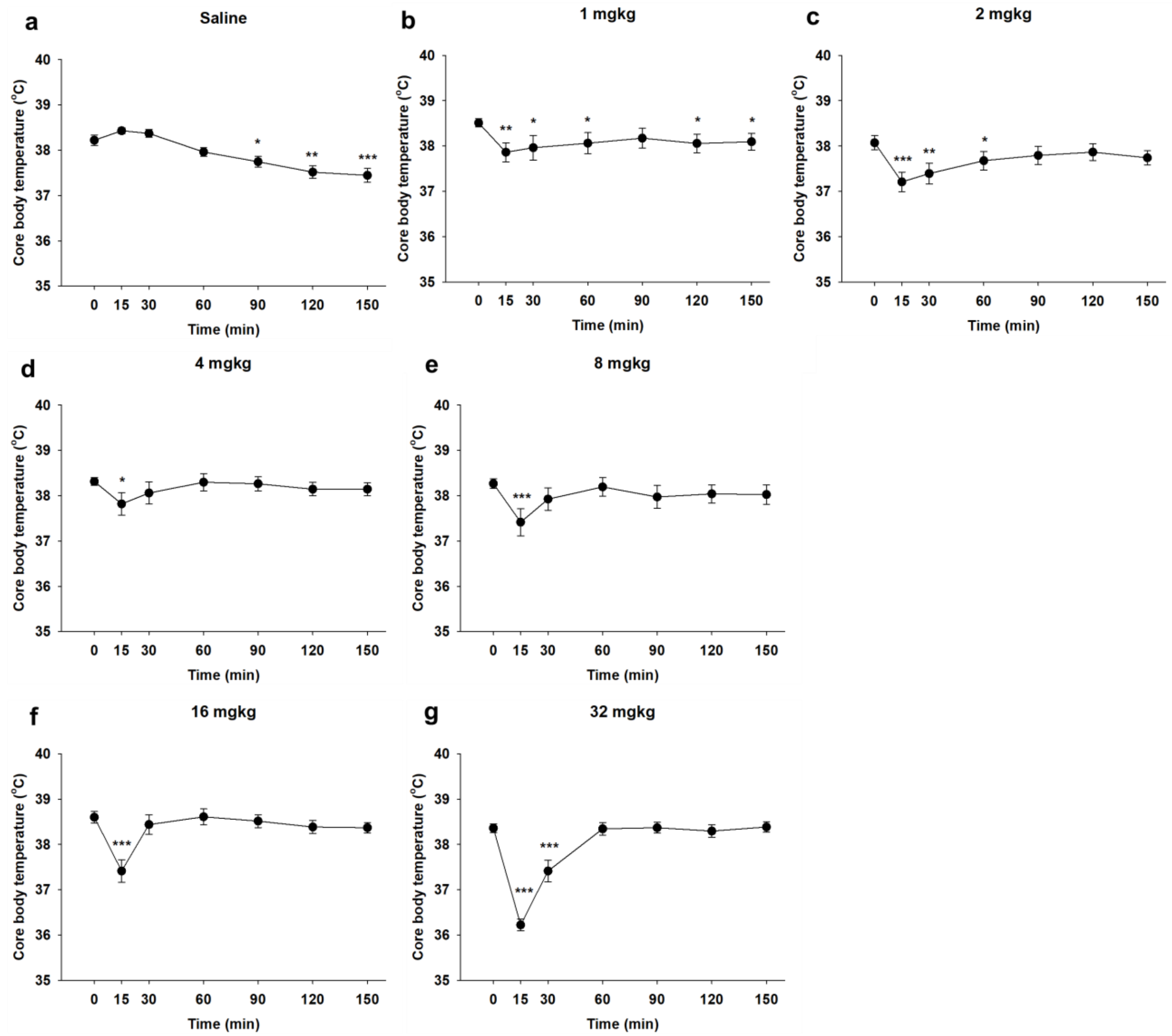

Figure 3.8. Buprenorphine induces dose-dependent hypothermia in mice bred for high methamphetamine (MA) intake. Shown are the effects of (a) saline, (b) 1 mg/kg, (c) 2 mg/kg, (d) 4 mg/kg, (e) 8 mg/kg, (f) 16 mg/kg, (g) 32 mg/kg buprenorphine on body temperature in MAHDR and MALDR mice across time in minutes (min). Data are means ± SEM. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ for temperature change from T0 at the indicated time point. MAHDR: methamphetamine high drinking

Buprenorphine: genotyping results and correlations in MADR mice

The genotyping results for *Taar1* and *Oprm1* in the MADR lines that received buprenorphine are summarized in Figure 3.9. MAHDR mice were almost exclusively *Oprm1*$^{D2/D2}$, with only one *Oprm1*$^{D2/B6}$ mouse, whereas MALDR mice were predominantly *Oprm1*$^{B6/B6}$ and *Oprm1*$^{D2/B6}$; no MAHDR mice possessed the *Oprm1*$^{B6/B6}$ genotype. For *Taar1*, MAHDR mice were exclusively *Taar1*$^{m1J/m1J}$ and MALDR mice were predominantly *Taar1*$^{+/+}$, with only 2 MALDR mice possessing the *Taar1*$^{+/m1J}$ genotype. Chi-square tests compared the observed genotype frequency ratio to the expected 1 : 2 : 1 ratio for the 4 possible combinations of *Oprm1* and *Taar1* genotype, had there been no impact of selective breeding on the frequency of *Oprm1* or *Taar1*. Results indicated that the observed ratios differed significantly from the expected ratios for both the MAHDR (chi-squared = 406.04 and 414.0 for *Oprm1* and *Taar1*, respectively, ps < 0.001) and MALDR (chi-squared = 166.89 and 410.17 for *Oprm1* and *Taar1*, respectively, ps < 0.001) lines. Similar to the morphine-treated cohort, there was a significant correlation between progenitor source of *Oprm1* and *Taar1* ($r = 92$, $p < 0.001$). *Taar1* genotype predicted *Oprm1* genotype approximately 86% of the time, again demonstrating linkage disequilibrium between these genes.

Correlations were separately calculated between thermal response to buprenorphine and *Oprm1* or *Taar1* genotype, and are presented in Table 3.1. Consistent with the morphine treatment genotype analysis, there were not enough mice of every *Oprm1* and *Taar1* genotype combination to assess correlations with allele combinations. Because maximum hypothermia was reached at T15, a change value (T15-T0) was calculated for each animal and used to assess correlations with genotype at each dose. For the 4 mg/kg dose there were significant correlations with *Oprm1* ($r = 0.40$, $p < 0.05$) and *Taar1* ($r = 0.41$, $p < 0.01$) genotype, and for the 32 mg/kg dose there were significant correlations with *Oprm1* ($r = -0.42$, $p < 0.01$) and *Taar1* ($r = -0.37$, $p < 0.05$) genotype. Temperature change at T15 and genotype were not significantly correlated at any other dose.

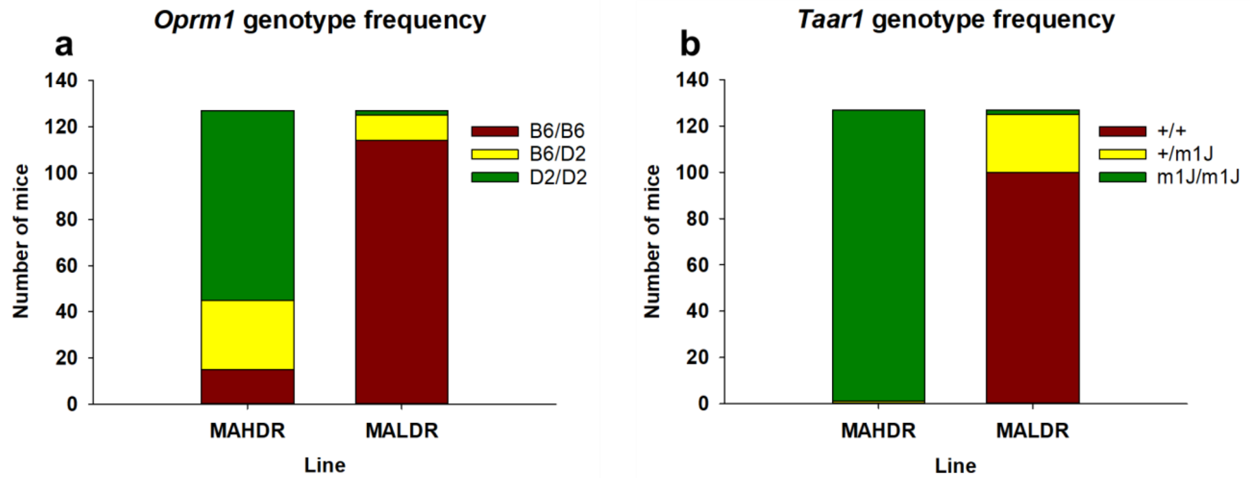

Figure 3.9. (a) *Oprm1* and (b) *Taar1* genotype frequencies for methamphetamine high and low drinking mice tested in the study of buprenorphine thermal effects. +: reference *Taar1* allele; B6: C57BL/6J; D2: DBA/2J; *m1J*: mutant *Taar1* allele found only in D2 mice; MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking; *Oprm1*: mu-opioid receptor gene; *Taar1*: trace amine-associated receptor 1 gene

Table 3.1. Pearson correlations between *Taar1* genotype or *Oprm1* genotype and temperature change. Bolded values indicate significant correlations ($p < 0.05$)

MODEL	DRUG	DOSE	GENE	*R*
MAHDR/MALDR REP 5	Saline	-	*Taar1*	0.00
MAHDR/MALDR REP 5	Morphine	15 mg/kg	*Taar1*	**0.48**
MAHDR/MALDR REP 5	Morphine	30 mg/kg	*Taar1*	**0.43**
MAHDR/MALDR REP 5	Saline	-	*Taar1*	0.06
MAHDR/MALDR REP 5	Morphine	15 mg/kg	*Oprm1*	**0.50**
MAHDR/MALDR REP 5	Morphine	30 mg/kg	*Oprm1*	**0.44**
BXD RI	Saline	-	*Taar1*	0.05
BXD RI	Morphine	15 mg/kg	*Taar1*	0.07
BXD RI	Morphine	30 mg/kg	*Taar1*	0.08
BXD RI	Saline	-	*Oprm1*	0.12
BXD RI	Morphine	15 mg/kg	*Oprm1*	**0.49**
BXD RI	Morphine	30 mg/kg	*Oprm1*	**0.63**
MAHDR/MALDR REP 5	Saline	-		0.08
MAHDR/MALDR REP 5	Buprenorphine	1 mg/kg	*Taar1*	0.04
MAHDR/MALDR REP 5	Buprenorphine	2 mg/kg	*Taar1*	0.11
MAHDR/MALDR REP 5	Buprenorphine	4 mg/kg	*Taar1*	**0.41**
MAHDR/MALDR REP 5	Buprenorphine	8 mg/kg	*Taar1*	0.05
MAHDR/MALDR REP 5	Buprenorphine	16 mg/kg	*Taar1*	0.19
MAHDR/MALDR REP 5	Buprenorphine	32 mg/kg	*Taar1*	**0.37**
MAHDR/MALDR REP 5	Saline	-	*Oprm1*	0.03
MAHDR/MALDR REP 5	Buprenorphine	1 mg/kg	*Oprm1*	0.02
MAHDR/MALDR REP 5	Buprenorphine	2 mg/kg	*Oprm1*	0.16

MAHDR/MALDR REP 5	Buprenorphine	4 mg/kg	*Oprm1*	**0.40**
MAHDR/MALDR REP 5	Buprenorphine	8 mg/kg	*Oprm1*	0.12
MAHDR/MALDR REP 5	Buprenorphine	16 mg/kg	*Oprm1*	0.12
MAHDR/MALDR REP 5	Buprenorphine	32 mg/kg	*Oprm1*	**0.42**

Discussion

Our findings indicate that genetic risk for MA intake in the MADR mouse lines is tied to thermal response to another amphetamine-like drug, MDMA. The lines do not differ in thermal response to cocaine or ethanol (Harkness et al., 2015), but, similar to previous results for MA (Harkness et al., 2015), MALDR mice exhibit dose-dependent hypothermia to the amphetamine-like stimulant, MDMA, whereas MAHDR mice are insensitive to MDMA-induced hypothermia. Overall, saline-treated mice had a decrease in body temperature over time, which can be attributed to loss of body heat due to isolate housing (Fantegrossi et al., 2003). *Taar1* impacts sensitivity to the hypothermic effect of MA (Harkness et al., 2015; Reed et al., 2017; Stafford et al., 2019) and the current results are consistent with a similar role for *Taar1* in sensitivity to the hypothermic effect of MDMA. The MADR lines differ in thermal response to the opioid, morphine. However, their sensitivity order is reversed, so that MAHDR mice exhibit a larger dose-dependent hypothermic response than MALDR mice. Our analysis in the BXD RI mice indicates that *Oprm1* genotype, rather than *Taar1* genotype, is associated with sensitivity to the hypothermic effect of morphine, and we identified linkage disequilibrium for *Oprm1* and *Taar1* in the MADR lines that likely accounts for their differential sensitivity to morphine-induced hypothermia. The partial OPRM1 agonist buprenorphine also induces hypothermia in both MAHDR and MALDR line mice, but the magnitude of sensitivity was sex-dependent. Certain lower doses of buprenorphine produced greater hypothermia in MALDR line mice than MAHDR line mice, but this was reversed at the highest dose. In contrast, among females MAHDR line mice were more sensitive to the hypothermic effects of lower doses of buprenorphine but the lines did not differ at higher doses.

Cocaine, MDMA, and MA all directly affect dopamine (DA) systems. However, MDMA and MA have effects that are distinct from cocaine. Amphetamine-like substances (e.g., MDMA and MA) are DA transporter (DAT) substrates, entering the presynaptic cell via DAT, facilitating the vesicular release of DA in the cytosol, and increasing extracellular DA via reverse transport

at DAT (Fleckenstein et al., 2007; Kahlig et al., 2005; Sulzer et al., 1995; Sulzer et al., 2005). Cocaine is a DAT inhibitor, interfering with DA uptake by DAT, causing a buildup of DA in the synapse (Jones, Garris, & Wightman, 1995; Krueger, 1990). In all cases, there is more DA available for receptor stimulation. However, MDMA and MA are also TAAR1 agonists, whereas cocaine is not (Bunzow et al., 2001; Simmler et al., 2016). Cocaine elicited a dose-dependent thermal response in both MADR lines, but the lines did not differ in this response. Similarly, the MADR lines do not differ in sensitivity to cocaine-conditioned reward or aversion, or locomotor stimulation (Gubner et al., 2013), whereas they do differ for these traits in relation to MA (Phillips & Shabani, 2015; Shabani et al., 2011; Shabani, Dobbs, et al., 2012; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). All of these findings suggest distinct mechanisms impact these responses after treatment with cocaine vs. MA in the MADR mouse lines, and one important factor is *Taar1* genotype.

We have determined that *Taar1* is a quantitative trait gene for MA intake, and also impacts sensitivity to the hypothermic effect of MA (Harkness et al., 2015; Shi et al., 2016; Alexandra M Stafford et al., 2019). Because the *Taar1*^{m1J} mutation codes for a nonfunctional receptor, the MAHDR line is a naturally occurring functional knockout. In the presence of functional TAAR1, MA and MDMA induce hypothermia at lower doses and hyperthermia at higher doses, whereas only hyperthermia occurs in response to some doses of these drugs in the absence of functional TAAR1 (Di Cara et al., 2011; Fantegrossi, Gannon, Zimmerman, & Rice, 2013; Harkness et al., 2015; Miner, Elmore, et al., 2017; Miner, O'Callaghan, et al., 2017; Reed et al., 2017). This indicates that different biological mechanisms drive these 2 types of thermal response. Since cocaine is not a TAAR1 agonist, yet reduces body temperature similarly in the MADR lines, cocaine must elicit its hypothermic effects via a TAAR1-independent mechanism not impacted by selective breeding for MA intake.

MAHDR mice are more sensitive to the hypothermic effect of morphine, and the MAHDR line also voluntarily consumes less morphine than the MALDR line (Eastwood & Phillips,

2014a). Thus, a negative relationship between drug-induced hypothermia and drug intake has been found for both morphine and MA in the MADR lines, suggesting there may be common genetic factors that influence the hypothermia and intake traits for each drug. For both MA and morphine, hypothermia may also increase the period during which negative associations can be conditioned (Misanin et al., 2002); thus the greater hypothermia experienced by MAHDR mice following morphine exposure may facilitate associations with the negative subjective effects of morphine. *Taar1* has a pleiotropic effect on MA-induced hypothermia and intake (Eastwood & Phillips, 2014a; Reed et al., 2017), and the current data suggest that *Oprm1* impacts morphine-induced hypothermia. We found that *Oprm1* alleles from the D2 and B6 progenitor strains exist at different frequencies in the MAHDR vs. MALDR lines and this appears to be due to linkage disequilibrium, rather than because *Oprm1* impacts risk for MA intake (Belknap et al., 2013; Eastwood et al., 2018). Thus, the $Oprm1^{D2}$ allele is linked to the $Taar1^{m1J}$ allele and occurs at higher frequency in MAHDR mice and the $Oprm1^{B6}$ allele is linked to $Taar1^{+}$ allele and occurs at higher frequency in MALDR mice. The progenitor D2 strain consumes less morphine and exhibits greater morphine-induced hypothermia, than the progenitor B6 strain (Belknap et al., 1989; Belknap, Crabbe, & Young, 1993; Doyle et al., 2008, 2014; Gora-Maslak et al., 1991; Horowitz, Whitney, Smith, & Stephan, 1977; Takamura Muraki & Ryuichi Kato, 1987), consistent with the *Orpm1* genotypes and morphine phenotypes of the MAHDR (largely $Oprm1^{D2}$; less morphine intake; greater morphine-induced hypothermia) and MALDR (largely $Oprm1^{B6}$; more morphine intake; less morphine-induced hypothermia) lines.

Compared to morphine, which produced clear line-dependent hypothermia, buprenorphine produced noticeably less predictable hypothermia, the magnitude of which was line-, sex-, and dose-dependent. We had two purposes for testing buprenorphine. First, we wished to explore the hypothermic effects of a full (morphine) vs partial (buprenorphine) OPRM1 agonist. We found substantial differences in the pattern of thermal responses produced by morphine and buprenorphine. Compared to morphine-induced hypothermia, which was

consistently greater in MAHDR line mice, buprenorphine-induced hypothermia was only greater in MAHDR line mice at the highest dose in males, and lower doses in females. Second, we wanted to test The results presented here support the hypothesis that 2mg/kg and 4mg/kg buprenorphine can induce hypothermia in MAHDR mice, and that these doses also reduce MA intake (Eastwood & Phillips, 2014a). However, when considering the hypothesis that the MAHDR line mice would be more sensitive to the hypothermic effects of buprenorphine, interpreting the hypothermia experienced by the MAHDR mice becomes more complicated. The interaction of sex, line, and buprenorphine dose permits the examination of the sexes separately, revealing that at lower doses male MAHDR appear resistant to buprenorphine-induced hypothermia. With that said there was no statistically significant difference in thermal response between male and female MAHDR mice. We cannot rule out buprenorphine-induced hypothermia as a cause of the decreased MA intake in MAHDR previously observed (Simmler et al., 2016). However, the hypothermia experienced by MAHDR was modest at the doses tested in conjunction with MA drinking (Eastwood & Phillips, 2014a). Furthermore, there were potential sex effects in the buprenorphine studies presented here, but no detectible sex effects when buprenorphine was tested for effects on MA intake (Eastwood & Phillips, 2014a).

Morphine is a full OPRM1 agonist and preferentially binds to ORPRM1, with approximately 2 and 85 times greater affinity for OPRM1 than for the kappa- and delta- opioid receptors, respectively (Gharagozlou, Demirci, Clark, & Lameh, 2002; Gharagozlou, Hashemi, DeLorey, Clark, & Lameh, 2006; Olson et al., 2019; Volpe et al., 2011), and functions as a weak partial agonist for these latter receptors as well (Gharagozlou et al., 2002, 2006; Olson et al., 2019; Volpe et al., 2011). Buprenorphine has higher affinity for OPRM1 than morphine, but is more promiscuous (Huang et al., 2001; Lutfy et al., 2003; Olson et al., 2019; Volpe et al., 2011). It reaches OPRM1 binding equilibrium at significantly lower concentrations than morphine (Huang et al., 2001; Lutfy et al., 2003; Olson et al., 2019; Volpe et al., 2011). But buprenorphine acts as an OPRM1 partial agonist (Olson et al., 2019). Morphine can achieve nearly complete

stimulation of OPRM1, whereas buprenorphine produces only about 1/3 maximum stimulation when compared to a positive control compound (Olson et al., 2019). Thus, buprenorphine produces its maximum stimulation of OPRM1 at very low concentrations in vitro (< 0.1 nM), but its maximum stimulation is substantially lower than that achieved by morphine. Buprenorphine is also a delta-opioid receptor partial agonist and kappa receptor antagonist, and a nociception receptor partial agonist (Huang et al., 2001; Lutfy et al., 2003; Olson et al., 2019; Volpe et al., 2011). With this in mind, there might be some explanation for the differences in thermal responses to buprenorphine and morphine reported here. If hypothermia produced by both drugs is dependent solely on OPRM1 activation, then one would expect buprenorphine to produce lesser maximal hypothermia relative to morphine in both lines, and any line differences should still be observable with both drugs. With that said, since buprenorphine is a partial agonist, hypothermic responses from it should follow a U-shaped curve, with increasing hypothermia as the dose increases until buprenorphine has reach its maximum efficacious dose, after which hypothermia should plateau, or even be reduced hypothermia as buprenorphine occupies OPRM1 preventing activation by endogenous ligands. However, here we found that increased doses of buprenorphine lead to a fairly linear increase in hypothermia. This indicates that either the doses used did not reach the maximum effective dose, or other OPRM1-independent mechanisms were mediating the thermal responses. Buprenorphine is substantially more potent at OPRM1 than morphine however (i.e. it reaches its maximum efficacy at much lower concentrations), so the doses required for it to produce maximum hypothermia should be lower than perhaps even morphine.

Differences in pharmacokinetics could also explain the thermal responses to buprenorphine and morphine. Morphine has a greater half-life in the brain relative to serum (about 45 min compared to about 30 minutes in rats, respectively) (Bouw, Gårdmark, & Hammarlund-Udenaes, 2000). In mice, the brain equilibrium half-life of sub-cutaneous morphine is approximately 78 minutes (Kalvass, Olson, Cassidy, Selley, & Pollack, 2007). This actually

corresponds with the morphine hypothermia we show here, which reaches its maximum between 60-90 minutes post-injection. In contrast, the serum half-life of sub-cutaneous buprenorphine is 23 hours, and the intravenous half-life is 3.7 hours (Kalliokoski, Jacobsen, Hau, & Abelson, 2011). These suggest significantly longer durations of action for buprenorphine than morphine, however here we observed buprenorphine hypothermia persisted for no longer than 90 min. If pharmacokinetics were a major contributor to differences in thermal responses to buprenorphine and morphine, then one would expect at least some correlation between brain or blood serum levels of each drug and thermal response to these drugs. However, this does not appear to be the case. These drastic discrepancies also indicate that these two d rugs do not produce hypothermia via the same mechanisms, since morphine corresponds with knowns half-lives and buprenorphine does not.

Opioid receptors in the brain and periphery can mediate hypothermic responses to opioids (Baker & Meert, 2002). OPRM1 agonists that do not cross the blood-brain-barrier induce hypothermia, which is attenuated in the by methylnaltrexone, a broad opioid receptor antagonist that does not cross the blood-brain-barrier (Baker & Meert, 2002; Yuan & Foss, 1999). However morphine-induced hypothermia is not affected by methylnaltrexone (Baker & Meert, 2002). Thus, morphine may produce hypothermia through peripherally and centrally located OPRM1 receptors, but since blocking peripheral opioid receptors does not affect morphine-induced hypothermia, the centrally mediated hypothermic effects of morphine must occlude any effects of peripheral OPRM1 activation. Additionally, both a non-selective opioid antagonist (naloxone) and an OPRM1 preferring antagonist produced similar reductions in morphine-induced hypothermia (Baker & Meert, 2002), indicating that morphine hypothermia is mediated largely by OPRM1 within the central nervous system. It may be the case that buprenorphine produces hypothermia through both OPRM1 pools (peripheral and central), but due to its low efficacy at activating OPRM1 the peripheral pool plays a somewhat larger role than the central pool, relative to morphine.

The story is further complicated when one considers research into OPRM1 and kappa-opioid receptors and thermoregulation. The literature largely agrees that kappa-opioid receptor agonists produce hypothermia, whereas OPRM1 activation generally produces hyperthermia (Cintron-Colon et al., 2019; Handler et al., 1992; Xin et al., 1997). Less is known about the role of delta-opioid receptors in thermoregulation, but their activation may mediate hypothermia (Rawls & Benamar, 2011). The thermoregulatory effects of morphine differ between rats and mice. In rats, morphine reliably produces hyperthermia until doses > 20 mg/kg (Chen et al., 1996; Geller et al., 1983; Rawls & Benamar, 2011). In mice however, ambient temperature plays a substantial role in this thermal response. At ambient temperatures below 25°C, doses of morphine > 10 mg/kg produce hypothermia (Baker & Meert, 2002; Rosow et al., 1980). In rats, higher doses of morphine induce hypothermia, which is due to off-target activation of kappa opioid receptors (Chen, Geller, Kim DeRiel, Liu-Chen, & Adler, 1996; Geller, Hawk, Keinath, Tallarida, & Adler, 1983; Rawls & Benamar, 2011). In mice off-target activation of kappa-opioid receptors cannot account morphine hypothermia, since morphine hypothermia in mice is only reversed by OPRM1 antagonists, not kappa- or delta-opioid receptor antagonists (Baker & Meert, 2002). One explanation is a difference in size and the ability of rats and mice to shed heat. Rats, due to their larger size, can depend on retention of body heat more than mice (Kleiber, 1932; Porter & Kearney, 2009). Since mice are smaller and already have a higher metabolic rate than rats, the increase in body temperature and their ability to retain heat may be fairly nominal in comparison to the greater disruptions in thermoregulation caused by morphine, the net result being an overall reduction in body temperature.

Buprenorphine is a kappa-opioid receptor antagonist (Huang et al., 2001; Lutfy et al., 2003; Olson et al., 2019; Volpe et al., 2011), so kappa-opioid receptor activation cannot explain the hypothermia we report. However, it is also a delta-opioid receptor agonist (Olson et al., 2019) so it is possible buprenorphine-induced hypothermia is mediated by delta-opioid receptor activation. Since it is an OPRM1 partial agonist, the degree of OPRM1 stimulation it produces

may not be high enough to produce measurable hyperthermia. These same mechanisms cannot explain the morphine hypothermia however, since morphine-induced hypothermia in mice is OPRM1-mediated.

Taken together some reasonable explanations for the patterns of buprenorphine and morphine hypothermia can be formed. Morphine is almost certainly producing hypothermia in the MADR line mice via activation of centrally located OPRM1. How this is possible is not known however. Buprenorphine could be producing hypothermia via the same mechanism, and/or through activation of delta opioid receptors and activation of peripherally located ORPM1. Buprenorphine produced less severe hypothermia, fitting with an OPRM1-mediated mechanism, but delta-opioid receptor activation could likewise produce less severe hypothermia. The fact that buprenorphine hypothermia did not asymptote suggests it may be mediated by delta-opioid receptors, then maximum possible stimulation was not achieved by the doses we used High doses of OPRM1 antagonists produce hypothermia, presumably by blocking endogenous opioids (Chen, McClatchy, Geller, Tallarida, & Adler, 2005). At the higher doses of buprenorphine we used, buprenorphine could be competing with endogenous opioids at OPRM1 and promoting hypothermia, which would also explain why we did not see buprenorphine hypothermia asymptote.

It should be acknowledged that morphine intake and buprenorphine effects on MA intake were measured in earlier replicates of MADR mice (Eastwood & Phillips, 2014a), and have not been evaluated in the replicates used here. We believe that it is likely that the same morphine-related outcomes would be obtained in all replicates of the MADR lines for the following reasons. First, the response to selection and differences between the MAHDR and MALDR lines for MA-related and several non-MA-related phenotypes have been highly reproducible across replicates (Harkness et al., 2015; Hitzemann et al., 2019; Phillips & Shabani, 2015; S. Shabani et al., 2011; Wheeler et al., 2009). Second, mapping results are consistent across replicates and support *Taar1* as a quantitative trait gene for MA intake in every replicate

(Belknap et al., 2013; Reed et al., 2017). Third, here we demonstrate linkage disequilibrium between *Taar1* and *Oprm1*, and thus the difference in *Oprm1*^{B6} *and Oprm1*^{D2} allele frequencies between the lines that we observed, likely occur in all replicates. Finally, published data from other labs for the B6 and D2 progenitors of the MADR lines are consistent with a negative correlation between morphine drinking and hypothermic response (Belknap et al., 1989; Belknap et al., 2013; Doyle et al., 2008, 2014; Gora-Maslak et al., 1991; Horowitz et al., 1977; Takamura Muraki & Ryuichi Kato, 1987). By not testing buprenorphine's thermal effects in BxD mice, we were not able to disassociate the relative contributions of *Taar1* and *Oprm1* genotype. Buprenorphine also did not have the same clear line-dependent hypothermic effects as morphine, making it difficult to attribute any effects we saw to *Taar1* and *Taar1*-linked genes. Because buprenorphine induced hypothermia in both lines, it is likely that similar results would be found in any replicate, since they all share the same progenitor strains.

Initial mapping suggested *Oprm1* as a candidate for our chromosome 10 MA intake QTL. However, fine mapping in existing chromosome 10 congenic strains derived from B6 and D2 mice (Doyle et al., 2008, 2014) excluded *Oprm1* as a genetic risk factor (Eastwood et al., 2018). Although not involved in risk for high MA intake, evidence from a gene expression microarray experiment using NAcc tissue from the MADR lines indicates that *Oprm1* is regulated by a gene expression network associated with risk for MA intake (Belknap et al., 2013), and *Taar1* and *Oprm1* interact to impact both MA intake and MA-induced hypothermia (Stafford et al., 2019). This led us to consider whether there might be an interaction between *Taar1* and *Oprm1* in their influence on the thermal response to morphine. Particular BXD RI strains were chosen for the current research to allow us to examine the independent and interactive effects of *Taar1* and *Oprm1* genotype on thermal response to morphine. *Oprm1*, but not *Taar1*, genotype was supported as a contributor. Mice with the *Oprm1*$^{D2/D2}$ genotype displayed greater hypothermia than mice with the *Oprm1*$^{B6/B6}$ genotype, but there was no correlation with *Taar1* genotype. However, in a recent exploration, *Oprm1* and *Taar1* were

found to have interactive effects on MA intake and thermal response to MA (Alexandra M Stafford et al., 2019). BXD RI mice with the *Taar1*$^{m1J/m1J}$ genotype consumed significantly more MA, and MA intake was synergistically enhanced in mice with the *Oprm1*$^{D2/D2}$ genotype. *Taar1*$^{+/+}$ BXD RI mice exhibited a hypothermic response to MA which was also synergistically enhanced in mice with the *Oprm1*$^{D2/D2}$ genotype. Correlational data for the BXD RI mice indicate that *Oprm1* genotype accounted for 24% and 40% of the variance in hypothermic response to the 15 and 30 mg/kg morphine doses. However, genes linked to *Oprm1* could impact morphine intake and some of the genes near *Oprm1* are known to be involved in the actions or effects of opioids, such as regulation of μ-opioid receptor signaling (Doyle et al., 2014). We cannot rule out a potential role for these genes in the current results for morphine-induced hypothermia.

Some evidence indicates a role for human *OPRM1* variants in risk for opioid and MA use (Deb, Chakraborty, Gangopadhyay, Choudhury, & Das, 2010; Ide et al., 2004; Jones et al., 2019). These variants may confer differences in receptor function, which may in turn contribute to risk for opioid use. Investigations into a QTL for differences in morphine preference between the B6 and D2 strains identified *Oprm1* as a candidate gene (Doyle et al., 2008, 2014; Horowitz et al., 1977). Several *Oprm1* polymorphisms exist between these strains that may result in functional differences (Doyle et al., 2014), which could impact receptor function and may explain the differences in thermal responses presented here. Future studies should examine the binding affinity and kinetics of opioids at these receptor variants.

The BXD RI mice could be enlisted to explore whether *Taar1* plays a role in the differences in morphine intake between the MADR lines and whether *Oprm1* genotype impacts the buprenorphine-induced reduction in MA intake that we previously reported in MAHDR mice (Eastwood & Phillips, 2014a). If *Oprm1* genotype, and not *Taar1* genotype, determines morphine intake, then *Oprm1*$^{B6/B6}$ mice should consume more morphine than *Oprm1*$^{D2/D2}$ mice, regardless of *Taar1* genotype. Groups of BXD RI mice, all with the *Taar1*$^{m1J/m1J}$ genotype to induce MA intake, but with either of the 2 *Oprm1* genotypes, could be used to determine if the

buprenorphine effect is dependent on *Oprm1*$^{D2/D2}$ vs. *Oprm1*$^{B6/B6}$ genotype, or if a reduction in MA intake occurs regardless of *Oprm1* genotype. The latter result could suggest that the reduction in MA intake involves μ-opioid receptor-independent mechanisms.

Chapter 4:

The glutamate system in a model of high genetic risk for methamphetamine intake

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Abstract

The glutamate system is necessary for learning and memory. Dysregulation of glutamate signaling is proposed as a common mechanism driving drug addiction, including methamphetamine (MA) addiction. The MA drinking (MADR) lines were developed to understand genetic and biological contributions to MA addiction. The high drinking line (MAHDR) differed in their glutamate system compared to the low drinking (MALDR) line. MAHDR line mice had elevated basal glutamate, and a greater increase in glutamate to a challenge dose of MA, within the nucleus accumbens (NAcc) relative to the glutamate levels MALDR line mice. Furthermore, MAHDR line mice had higher levels of the metabotropic glutamate receptor 5 (mGLuR5) and the scaffolding protein Homer2, and lower levels of Homer1 and the neuronal glutamate transporter (EAAT3), also within the NAcc. There are also differences in medial prefrontal cortex (mPFC) glutamate. MAHDR line mice have decreased Homer2 and mGluR2. They also have higher extracellular glutamate but display a decrease in extracellular glutamate when MA is injected, which is not observed in MALDR line mice. These data suggest glutamate dysregulation could contribute to the high MA intake of MAHDR line mice. Here, I determine the efficacy of drugs targeting the glutamate system to attenuate MA intake in the MAHDR line. I tested drugs affecting three glutamate system targets that have been implicated in addiction-related behaviors, the metabotropic glutamate receptors 2 and 5 (mGluR2 and mGluR5), and the cystine-glutamate antiporter (x_c^-). The drugs tested, an mGluR2 positive allosteric modulator, two mGluR5 negative allosteric modulators, and a x_c^- prodrug, were incapable of altering the acquisition of MA intake, nor were the mGluR5 allosteric modulators capable of altering established MA intake. I then measure levels of glutamate-related proteins within the NAcc of MAHDR and MALDR line mice. The lines did not differ in expression of mGluR5, Homer2a/b, the glutamate transporters EAAT3 or EAAT2, x_c^-, or the vesicular glutamate transporter 1 (VGLUT1). Thus, although glutamate may have contributed to the MA intake differences of earlier replicates of MADR lines, it may not play as large a role in

the replicate tested here. Furthermore, since drugs intended to reduce glutamatergic signaling failed to alter MA intake of MAHDR line mice, such drugs may not be effective pharmacotherapies, especially for those with high genetic risk for MA addiction.

Introduction

With a continuing increase in methamphetamine (MA) use and MA-related deaths (Han, Compton, et al., 2021; Han, Cotto, et al., 2021) comes a need to improve treatment options. Behavioral therapies are the most common interventions, however relapse is common (AshaRani et al., 2020; De Crescenzo et al., 2018; Hamel et al., 2020; Paulus & Stewart, 2020). Pharmacological interventions are restricted to drugs targeting symptoms of withdrawal, or underlying mental health conditions (AshaRani et al., 2020; De Crescenzo et al., 2018; Hamel et al., 2020; Paulus & Stewart, 2020). An increased understanding of the biological mechanisms underlying MA addiction offers the potential for more targeted interventions that may improve outcomes.

The MA drinking (MADR) lines were developed to improve understanding of the genetic, and mechanistic factors contributing to MA intake risk. They were selectively bred for high (MAHDR) and low (MALDR) MA intake from an F2 cross of the C57BL/6J (B6) and DBA/2J (D2) progenitor strains (Shabani et al., 2011; Wheeler et al., 2009). On average, MAHDR line mice display binge-like voluntary MA intake (Shabani et al., 2016; Wheeler et al., 2009), and resistance to aversive effects of MA (Phillips & Shabani, 2015; Shabani, Dobbs, et al., 2012; Shabani, McKinnon, et al., 2012). In contrast, MALDR line mice are sensitive to the aversive effects of MA, and voluntarily consume virtually no MA (Phillips & Shabani, 2015; Shabani et al., 2011; Shabani, Dobbs, et al., 2012; Wheeler et al., 2009). Thus the MAHDR line is a model of genetic risk for high MA intake, and the MALDR line is a model for genetic protection against MA intake (Phillips & Shabani, 2015; Shabani et al., 2011; Shabani, Dobbs, et al., 2012; Shabani et al., 2016; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). In exploring the genetic origins of these differences, it was revealed that the trace amine associated receptor 1 gene (*Taar1*) is a quantitative trait gene that accounts for 60% of the genetic variance in MA intake in these lines (Harkness et al., 2015; Phillips & Shabani, 2015; Stafford et al., 2019). The D2 progenitor strain contributes a mutant *Taar1* allele ($Taar1^{m1J}$) that codes for a nonfunctional

receptor (TAAR1) (Harkness et al., 2015; Shi et al., 2016). There is dominance for the alternative *Taar1* allele (*Taar1*$^{+}$) contributed by the B6 progenitor strain, which expresses a functional TAAR1. The MAHDR line is homozygous for *Taar1*^{m1J} and therefore expresses only nonfunctional TAAR1 (Harkness et al., 2015; Reed et al., 2017). The MALDR line possesses at least one copy of the alternative allele and therefore expresses functional TAAR1 receptors (Harkness et al., 2015). Functional TAAR1 serve as regulators of monoamine release (Revel et al., 2011; Xie & Miller, 2008; Xie & Miller, 2009b), and more recent work indicates that TAAR1 also are involved in regulating synaptic glutamate (Underhill et al., 2019; Underhill, Ingram, Ahmari, Veenstra-VanderWeele, & Amara, 2018). In this capacity, TAAR1 regulates the trafficking of the neuronal glutamate transporter (EAAT3) (Underhill et al., 2019, 2018). MA, a TAAR1 agonist, impacts EAAT3 trafficking (Underhill et al., 2019, 2018).

The consequences of TAAR1 variants on glutamate transmission have not fully been elucidated, but it is safe to assume there is some disruption. In addition, within the nucleus accumbens (NAcc) core and shell of MA-naïve MADR mice, MAHDR mice express elevated levels of the metabotropic glutamate receptor 5 (mGluR5) and the scaffolding protein Homer2a/b, and within the NAcc core alone they have decreased EAAT3 and Homer1b/c (Szumlinski et al., 2017). They also have higher baseline glutamate, and a greater increase in glutamate to a challenge dose of MA (Szumlinski et al., 2017). Within the medial prefrontal cortex (mPFC), relative to MALDR line mice, MAHDR mice have higher extracellular glutamate, but achieve a significant reduction in glutamate after a challenge dose of MA (Lominac et al., 2016). Also within the mPFC they have decreased Homer2a/b and mGluR2 levels (Szumlinski et al., 2017).

The potential importance of these differences becomes clear when considering the role of glutamate in drug addiction. Kalivas (2009) synthesizes much of this research, outlining a glutamate homeostasis hypothesis of addiction. This glutamate homeostasis hypothesis postulates that varying mechanisms of glutamate regulation are perturbed by addictive drugs,

leading to dysregulation of appropriate behaviors after chronic drug use (Kalivas, 2009). It is grounded in the principle that maladaptive drug-seeking is an inability to properly inhibit drug-seeking behaviors governed by a corticostriatal circuit (Barnes, Kubota, Hu, Jin, & Graybiel, 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Kalivas, 2009; McClure et al., 2014; Robbins & Everitt, 2002; Vandaele & Ahmed, 2021; Wise & Koob, 2014; Yin & Knowlton, 2006). In a simplified view, this circuit is comprised of 2 subcircuits; the limbic subcircuit consisting of the PFC, NAcc, and ventral tegmental area (VTA); and the motor subcircuit consisting of the motor cortex, substantia nigra, the dorsal striatum, and other basal ganglia nuclei (Barnes et al., 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Kalivas, 2009; Robbins & Everitt, 2002; Yin & Knowlton, 2006). The limbic subcircuit processes new information, relating it to previous experiences and learned behaviors, and modifies the output behavior to adapt to stimuli (Balleine, Liljeholm, & Ostlund, 2009; Barnes et al., 2005; Kalivas, 2009; Yin & Knowlton, 2006; Yin, Ostlund, & Balleine, 2008). The motor subcircuit is responsible for behavioral outputs (Barnes et al., 2005; Kalivas, 2009; Yin & Knowlton, 2006). In the presence of novel stimuli, recruitment of the limbic subcircuit is prioritized, modifying behaviors to best respond to the stimuli (Balleine et al., 2009; Furlong, Corbit, Brown, & Balleine, 2018; Kalivas, 2009; Yin & Knowlton, 2006; Yin et al., 2008). As a behavior yields consistent results, less reliance is placed on the limbic subcircuit and more on the motor subcircuit (Barnes et al., 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Gardner, 2011; Kalivas, 2009; Lüscher, Robbins, & Everitt, 2020; Robbins & Everitt, 2002; Swanson, 2000; Vanderschuren & Everitt, 2004; Wise & Koob, 2014; Yin & Knowlton, 2006). If the outcome of a previous learned behavior changes, the limbic subcircuit is reengaged and the behavior is modified if necessary (Barnes et al., 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Gardner, 2011; Kalivas, 2009; Lüscher et al., 2020; Robbins & Everitt, 2002; Swanson, 2000; Vanderschuren & Everitt, 2004; Wise & Koob, 2014; Yin & Knowlton, 2006). Thus, the limbic subcircuit updates behaviors, and the motor subcircuit establishes behaviors. Within this circuit the NAcc plays a unique role, acting as a “gateway”

between the limbic and motor subcircuits, allowing for the necessary influence of the limbic subcircuit over the motor subcircuit (Gardner, 2011; Kalivas, 2009; Noori, Spanagel, & Hansson, 2012; Quintero, 2013; Yin et al., 2008). Glutamatergic afferents to the NAcc are responsible for encoding the contingencies associated with a specific situation (Britt et al., 2012; Kalivas, 2009; Kelley, 2004; Moussawi et al., 2009; Salgado & Kaplitt, 2015). Repeated drug use disrupts homeostasis of glutamate within the NAcc, impairing the ability of the limbic subcircuit to integrate novel information about behavioral outcomes and modify learned behaviors (Britt et al., 2012; Kalivas, 2009; Kelley, 2004). Consequently, drug-related behaviors become compulsive (Barnes et al., 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Gardner, 2011; Kalivas, 2009; Lüscher et al., 2020; Robbins & Everitt, 2002; Swanson, 2000; Vanderschuren & Everitt, 2004; Wise & Koob, 2014; Yin & Knowlton, 2006).

There are numerous changes to the glutamate system in response to drug exposure. However, relating directly to pivotal PFC glutamatergic projections to the NAcc, Kalivas (Kalivas, 2009) highlights a trio of adaptations to glutamate regulation and signaling that present promising treatment targets. These adaptations involve the cystine-glutamate antiporter (x_c^-), the metabotropic glutamate receptor 2 (mGluR2), and mGluR5. x_c^- is a Na^+-independent transporter that exchanges extracellular cystine for intracellular glutamate (Baker, Xi, Shen, Swanson, & Kalivas, 2002; Bannai, 1986; Danbolt, 2001; McBean, 2002). Within the central nervous system, it is primarily expressed in glia (Baker et al., 2002; Danbolt, 2001; McBean, 2002; Pow, 2001), and is responsible for approximately 60% of the extracellular glutamate in the NAcc (Baker et al., 2002). Glutamate originating from x_c^- transport stimulates the G_i-coupled mGluR2 and the $G_{q/11}$-coupled mGluR5 in the NAcc (Kalivas, 2009; Kupchik et al., 2012; Moran et al., 2005; Moussawi et al., 2009; Zheng-Xiong Xi et al., 2002), where mGluR2 acts as an autoreceptor or heteroreceptor, thereby inhibiting glutamate release (Conn & Pin, 1997; Lovinger & McCool, 1995; Niswender & Conn, 2010; Xi et al., 2002). Group II mGluRs, which include mGluR2, are also a source of NMDAR-independent long-term potentiation (LTP)

(Grover & Yan, 1999; Kalivas, 2009; Wu et al., 2004). In the NAcc, mGluR5 is predominantly expressed in postsynaptic elements (Mitrano & Smith, 2007; Shigemoto et al., 1993), and induces long-term depression (Conn & Pin, 1997; Kalivas, 2009; Malenka & Bear, 2004; Nicoletti et al., 2011). mGluR5 is downregulated during withdrawal from cocaine (Ghasemzadeh, Vasudevan, Mueller, Seubert, & Mantsch, 2009) and mGluR5-dependent LTD is reduced (Kasanetz et al., 2010; Lüscher & Huber, 2010). Membrane-bound levels of x_c^- are measurably reduced after nicotine and cocaine self-administration (Kalivas, 2009; Kau et al., 2008; Knackstedt et al., 2009; Knackstedt, Melendez, & Kalivas, 2010; Madayag et al., 2007; Pendyam, Mohan, Kalivas, & Nair, 2009), thereby reducing extrasynaptic glutamate. Typically, mGluR2 would be activated by extrasynaptic glutamate and attenuate glutamate signaling (Baker et al., 2003; Kupchik et al., 2012; Xi et al., 2002). But after repeated drug exposure, x_c^- derived extrasynaptic glutamate is greatly reduced, as is mGluR2 membrane expression, permitting persistent potentiation that occludes LTP (Bowers et al., 2004; Ghasemzadeh et al., 2009; Liechti, Lhuillier, Kaupmann, & Markou, 2007; Moussawi et al., 2009; Xi, 2002). Activating mGluR2 restores LTP and inhibits cocaine, heroin, nicotine, and alcohol seeking (Acri, Cross, & Skolnick, 2017; Augier et al., 2016; Baptista, Martin-Fardon, & Weiss, 2004; Bossert, Gray, Lu, & Shaham, 2006; Jin et al., 2010; Liechti et al., 2007; Niedzielska-Andres et al., 2021; Peters & Kalivas, 2006), and reduces nicotine, cocaine, alcohol, and MA self-administration (Acri et al., 2017; Augier et al., 2016; Bäckström & Hyytiä, 2005; Crawford, Roberts, & Beveridge, 2013; Liechti et al., 2007; Niedzielska-Andres et al., 2021). The changes in mGluR5 expression and mGluR5-dependent LTD are actually believed to be compensatory for a number of reasons (Kalivas, 2009; Moussawi et al., 2009). For instance, mGluR5 null mutant mice do no self-administer cocaine (Christian Chiamulera et al., 2001). Additionally mGluR5 antagonists inhibit ethanol, cocaine, nicotine, opiate, amphetamine and MA seeking and self-administration (Brown et al., 2012; Gass et al., 2009; Herrold et al., 2013; Hodge et al., 2006; Niedzielska-Andres et al., 2021; Osborne & Olive, 2008; Palmatier et al., 2008; Tronci, Vronskaya, Montgomery, Mura,

& Balfour, 2010), and mGluR5 activation enhances cocaine seeking (Kalivas, 2009; Moussawi et al., 2009). Application of n-acetylcysteine (NAC), a cystine prodrug, restores some of the x_c^- derived extrasynaptic glutamate, and can reduce seeking and self-administration of opioids, nicotine, and cocaine (Baker et al., 2003; Bridges, Lutgen, Lobner, & Baker, 2012; Kalivas, 2009; Knackstedt et al., 2009; LaLumiere & Kalivas, 2008; McClure et al., 2014; Moran et al., 2005; Powell et al., 2019). NAC has also been proposed as a treatment for drug addictions in humans (Grant, Odlaug, & Kim, 2010; McKetin et al., 2017; Mousavi et al., 2015).

Given the preponderance of data implicating glutamate dysregulation in addiction, the MAHDR line may have specific profile of mGluR expression and trafficking, and glutamate release and reuptake predisposing them to higher MA intake. As noted, MAHDR line mice display a hyperglutamatergic state within the NAcc, characterized by elevated baseline glutamate and an exaggerated glutamate response to MA (Szumlinski et al., 2017). Higher baseline glutamate within the NAcc is not consistently found after administration of MA or other addictive drugs (Scofield et al., 2016; Scofield; Hearing et al., 2017). In fact, lower baseline NAcc and PFC glutamate is found in animals experiencing MA withdrawal (Parsegian & See, 2014). However, across all drugs, an increase of accumbal glutamate is observed during cue and drug-primed reinstatement of drug seeking, including MA reinstatement (Scofield et al., 2016). MA-induced CPP is facilitated by inhibiting glutamate reuptake in the NAcc, and attenuated with the application of mGluR2/3 agonists (Szumlinski et al., 2017). Considering these data together, the high baseline accumbal glutamate of MAHDR line mice may not contribute to their MA intake, but their heightened glutamate response to MA could. Testing drugs that attenuate glutamate signaling in MAHDR line mice could elucidate the role of glutamate in their MA intake. Testing drugs in these mice also serves to assess the clinical applications of these drugs in a model of high genetic risk for MA addiction.

Facilitation of x_c^- glutamate release with NAC, activation of mGluR2, and mGluR5 antagonism, could all reduce MA stimulant self-administration and reinstatement. We

hypothesized that achieving these effects pharmacologically may also attenuate MA intake by MAHDR mice. We selected NAC as it is well-studied, increases extracellular glutamate, and decreases drug-seeking and self-administration (Baker et al., 2003; Bridges et al., 2012; Kalivas, 2009; Knackstedt et al., 2009; LaLumiere & Kalivas, 2008; McClure et al., 2014; Moran et al., 2005; Powell et al., 2019). We also selected the mGluR2 positive allosteric modulator (PAM) Biphenylindanone A (BINA), and the mGluR5 negative allosteric modulators (NAM) 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) and N-(5-Fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (VU0424238). Allosteric modulators were chosen as they are well-tested and offer more selectivity for specific mGluRs (Engers & Lindsley, 2013; May, Leach, Sexton, & Christopoulos, 2007). We tested the ability of mGluR5 NAMs to reduce established MA intake, as these drugs reduce acquired MA self-administration (Gass et al., 2009; Osborne & Olive, 2008). Testing for reductions in established intake also has more clinical relevance. We tested all drugs for their ability to attenuate the acquisition of MA intake. Since NAC is administered orally when tested in humans (Grant et al., 2010; McKetin et al., 2017; Mousavi et al., 2015), and oral self-administration is less disruptive to mice, we examined whether oral self-administration of this drug was sufficient to reduce the acquisition of MA drinking. We tested co-administration of NAC and MA, and chronic pretreatment of oral NAC. Chronic NAC injections can inhibit cocaine seeking (Reichel, Moussawi, Do, Kalivas, & See, 2011), and thus we felt it worthwhile to explore the possibility that enduring changes due to chronic oral NAC self-administration could inhibit MA drinking acquisition.

Finally, we tested whether an independent selection replicate of MADR mice recapitulated the differences in glutamate-related proteins from an earlier replicate (Szumlinski et al., 2017). We selected mGluR5, Homer2a/b, and EAAT3, as they were differentially expressed in the NAcc of the MADR lines (Szumlinski et al., 2017). We selected EAAT2 as a negative control as it was not previously differentially expressed (Szumlinski et al., 2017). We also selected proteins not previously examined. The novel proteins selected were xCT, the

unique catalytic subunit of x_c^- (Baker et al., 2002; Bannai, 1986; Danbolt, 2001; McBean, 2002), to explore whether or not there are differences in extrasynaptic glutamate regulation, and the vesicular glutamate transporter 1 (VGLUT1), as it is preferentially expressed in cortical neurons (Herzog et al., 2001) and could elucidate differences in the origins of NAcc glutamate.

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 Veterans Affairs Portland Health Care System (VAPORHCS) Institutional Animal Care and Use Committee. Prior to experimentation, all mice were group-housed (2-5 per cage) in polycarbonate shoebox cages (28.5 × 17.5 × 12 cm) with wire tops and Bed-O'Cobs bedding (The Andersons, Maumee, OH, USA). Mice had free access to rodent food (Purina 5001 or 5LOD PicoLab Rodent Diet; Animal Specialties, Woodburn, OR) and were maintained on a 12:12 hour light:dark cycle with lights on at 0600 hour. The colony room temperature was 21 ± 1 °C.

One hundred and thirty-two male and 132 female MAHDR and MALDR mice, aged 80-105 days old participated in these experiments. Numbers for each experiment are given below. All mice were experimentally naïve at the time of testing. Details of selective breeding have been published (Hitzemann et al., 2019; Shabani et al., 2011; Wheeler et al., 2009). Mice were from the 5th replicate of MADR lines from selection generations 1-5, varying across studies.

Drinking Procedure

Drugs

MA hydrochloride, NAC, and MTEP were obtained from Sigma (St. Louis, MO, USA). VU0424238 was generously gifted by Dr. Jeffrey Conn at Vanderbilt University. BINA was purchased from Axon Medchem (Groningen, Netherlands). MA was dissolved in tap water. MTEP and BINA were dissolved in 1% DMSO (Sigma-Aldrich, St. Louis, MO) in sterile saline (0.9% NaCl, Baxter Healthcare Corporation, Deerfield, IL), the lowest DMSO concentration

capable of dissolving both drugs and within a non-toxic range of DMSO (Galcao et al., 2014; Brown et al., 2012). VU0424238 was dissolved in 10% Tween 80 (Sigma-Aldrich, St. Louis, MO) in sterile saline. NAC was dissolved in sterile saline for injections, and in tap water for oral administration. Drug doses were chosen based on a literature review demonstrating behavioral effects in mice at these doses (Cozzoli et al., 2014; Brodkin et al., 2002; Cowen et al., 2007; Galici et al., 2006; Brown et al., 2012; Galici et al., 2006; Herrmann, Andrejew, Benvenutti, Gama, & Elisabetsky, 2018; Jin et al., 2010; Reichel et al., 2011). The concentration of NAC in drinking solutions was selected to achieve a desired dose of 200 mg/kg, which is higher than most bolus injections used (Charntikov, Pittenger, Pudiak, & Bevins, 2018; Herrmann, Benvenutti, Pilz, & Elisabetsky, 2014; Lebourgeois, González-Marín, Antol, Naassila, & Vilpoux, 2019; Reichel et al., 2011). Because the NAC is dissolved in drinking water, the 200 mg/kg dose would be achieved over a much longer period of time than a bolus injection. Average volume consumed for this calculation was based on intake during selective breeding of the fifth MAHDR replicate, which was 330±45 ml/kg/day (unpublished data).

Experiment 1: The effects of mGluR5 NAMs on established MA drinking

Two different NAMs were tested (MTEP and VU0424238) in MAHDR mice that had already established MA drinking. The two drugs were tested in two cohorts of mice, with nearly identical procedures, except for differences noted below. Drinking procedures were based on previously published studies (Eastwood & Phillips, 2014a).

MTEP

Forty-eight MAHDR mice participated in this study (6/sex/dose). One female died during the experiment and its data were excluded. Mice underwent a limited access 2-bottle choice MA drinking procedure. MA was offered for the first 6 hour of their dark cycle in order to maximize hourly MA intake. Two weeks prior to experimentation, mice were removed from the colony room into a separate experimental room, and placed on a reverse light:dark cycle (lights on at 0900 and off at 2100 hours). The experiment lasted 18 days. On day 1, mice were singly

housed. On days 1-2, mice were offered two 25 mL graduated cylinders filled with tap water. Fluid levels were recorded at 0900 and 1500 hours (6-hours into the dark cycle). On days 3-18, one water cylinder was replaced with a cylinder containing MA for the first 6 hour of the dark cycle (0900-1500), after which the MA-filled cylinder was removed and mice had access to one water cylinder for 18-hours (1500-0900). Fluid levels were measured at the beginning of the MA access period, every 2-hours during the MA access period, and at the end of the MA access period. This divided the MA access period into 3, 2-hour segments. The concentration of MA in the MA-filled cylinder was doubled every 4 days until the MA concentration was 80 mg/L. Thus, on days 3-6 mice were offered 20 mg/L MA, on days 7-10 mice were offered 40 mg/L MA, and on days 11-20 mice were offered 80 mg/L. The MADR lines were created using 20 and 40 mg/L concentrations, and bred based on average MA consumption for the higher concentration (Wheeler et al., 2009). The progression to 80 mg/L was designed to increase MA intake, as MAHDR mice consume more MA with increasing MA concentration (Shabani et al., 2016). The relative side of the MA and water cylinders was switched every 2 days to account for any side preference. On the same day cylinder sides were switched, mice were also weighed. On days 15-16, 30 min before the start of the dark cycle of each day, mice were injected with vehicle to familiarize them with handling and injections. On days 17-18, 30 minutes before the start of the dark cycle of each day, mice were injected with vehicle or their assigned dose of MTEP (0, 5, 10, or 20 mg/kg). The dose each mouse got was consistent for the duration of the study. To ensure that no treatment group (vehicle or each MTEP dose) differed based on subjects' innate MA or fluid intake, mice were assigned to treatment groups based on their intake from the 2 vehicle injection days (days 15-16).

VU0424238

Forty-eight MAHDR mice participated in this study (6/sex/dose). Two female mice died during the experiment and their data were excluded. Procedures were identical to those used to test MTEP, except that 10 mL serological pipettes custom fitted with metal sipper tops were

used instead of 25 mL graduated cylinders. This was done to increase accuracy of fluid measurements. Instead of MTEP, mice were injected with VU0424238 (0, 1, 3, or 10 mg/kg). Doses were chosen based on consultation with the lab of Dr. Jeffrey Conn from his unpublished data. Mice were assigned to a treatment group using the same methods and criteria as when testing MTEP.

Experiment 2: The effects of an mGluR2 PAM, mGluR5 NAM, and xc^- modulator on the acquisition of MA drinking

Forty-eight MAHDR mice participated in this study (6/sex/dose), and all 3 drugs were tested in this set of mice. The 3 drugs tested for their impact on the acquisition of MA intake were the mGluR2 PAM (BINA), the mGluR5 NAM VU0424238, and an xCT prodrug (NAC). The experiment lasted for 12 days. Six males died during the study and their data were excluded. This experiment utilized a similar 2-bottle choice procedure as in Experiment 1. The experimental room was under a 12-hour reverse light:dark cycle (lights off at 1100 hours), and mice were given the same acclimation time to this room as in Experiment 1. Mice were weighed every other day. Unlike Experiment 1, the relative side of the MA and water tubes did no change. The relative sides of these tubes were counterbalanced across all animals, and for a given animal did not change throughout the study, to increase the likelihood of obtaining stable drinking, as the animals would be certain of MA location.

See Table 4.1 for an experimental timeline. On day 1, mice were weighed and singly housed. Each day followed the same timeline. Starting at 1100 h, the beginning of the dark cycle, 2 10 mL tubes were placed on each cage. Mice had access to 2 drinking tubes for 4 hours each day. We reduced this access period from 6 hour to limit the possibility that mice would have access to MA after the effects of a pre-treatment drug had worn off. Since we were not changing the relative side of the MA and water tubes and MAHDR mice consume a stimulating dose of MA in the first 4 hours of MA access (Eastwood & Phillips, 2014a), this should provide adequate time for mice to consume a sufficient dose of MA to experience

psychoactive effects. Fluid levels were recorded at 1100, 1300, and 1500 h. At 1500 h, one tube was removed and mice had access to one water tube until the next day. On days in which MA was offered, it was the MA tube that was removed. On days in which 2 water tubes were offered, the water tube that was on the assigned side for the MA tube for a particular animal was removed. On days 1-2, two water tubes were offered. On days 3-4, 2 water tubes were offered, and mice were injected with vehicle immediately before access to the tubes began. This was done to familiarize mice with handling and injections. On days 5-12, during the 4-hour period when mice had access to 2 drinking tubes, one tube was filled with 20, then 80 mg/L of MA. Each concentration was offered for 4 days. On these days, immediately prior to MA access, mice were injected with the vehicle or the appropriate drug.

Table 4.1. Treatment group and general timeline for Experiment 2.

Group	Day 1-2: Drinking solution; Pre-treatment	Day 3-4: Drinking solution; Pre-treatment	Day 5-8: Drinking solution; Pre-treatment	Day 9-12: Drinking solution; Pre-treatment
Vehicle	Water; No Inj	Water; Vehicle	H_2O vs. 20 mg/L MA; Vehicle	H_2O vs. 40 mg/L MA; Vehicle
VU0424238	Water; No Inj	Water; Vehicle	H_2O vs. 20 mg/L MA; drug	H_2O vs. 40 mg/L MA; Drug
BINA	Water; No Inj	Water; Vehicle	H_2O vs. 20 mg/L MA; drug	H_2O vs. 40 mg/L MA; Drug
NAC	Water; No Inj	Water; Vehicle	H_2O vs. 20 mg/L MA; drug	H_2O vs. 40 mg/L MA; Drug

Experiment 3: The effects of oral NAC on MA intake

A pilot study confirmed mice would freely drink the concentration of NAC offered in the expected volumes (data not shown). We used 666 mg/L NAC in drinking solutions, which yielded a daily NAC dose of 160-180 mg/kg per day.

Forty-eight MAHDR mice participated in and completed this study (6/sex/experimental group). Experimental groups and an experimental timeline are found in Table 4.2. Animals were under a standard 12-hour light:dark cycle, with lights on from 0600-1800 hours. Mice had access to a single 25 mL graduated cylinder filled with tap water for 6 hours per day (0900-1500 hours). For the remaining 18 h, mice had access to 2 cylinders. The solutions in the cylinders during this period were dependent on group assignment and day of the study. On day 1, mice were weighed and isolate housed. On days 1 and 2, starting at 0900 h, mice were provided 1 cylinder filled with tap water. After 6 h, a second water cylinder was placed on the cage and mice had access to these 2 water cylinders for 18 h. This was done to acclimate mice to drinking from the cylinders. Mice were then divided into 4 groups (Table 4.2). These groups represented two NAC treatment methods. Groups 1 & 2 tested co-administration of MA and NAC, such that mice had access to NAC only while MA was present. Groups 3 & 4 represented pretreatment with NAC, such that mice orally self-administered NAC prior to any access to MA. It is important to highlight that if and when NAC was present, it was present in both available drinking tubes, even when the other tube contained MA as well. This was done to prevent mice from avoiding NAC and thus not receive a sufficient dose.

The experiment was divided into 2 phases, each lasting 8 days. During Phase 1, mice were given some combination of tap water, NAC solution, or MA+NAC solution, depending on experimental group. The solution in the cylinders were as follows: MA+NAC/NAC (Group 1), MA/water (Group 2), NAC/NAC (Group 3), and water/water (Group 4). These solutions were only available for the 18 hours encompassing the dark cycle and 3 hours before and 3 hours after the dark period; mice still received only 1 water cylinder for the 6-hour period during the

remaining hours of the light cycle (0900-1500 h). If mice were offered MA during Phase 1 (Groups 1 & 2), they were offered 20 mg/L MA, then 40 mg/L MA, for 4 days each.

During Phase 2, mice were offered only tap water or MA. NAC was never present during this phase. If mice were offered MA during Phase 1 (groups 1 & 2), during Phase 2 they were offered 40 mg/L MA for the entire phase. If mice were not offered MA during Phase 1, (Groups 3 & 4), during Phase 2 they were first offered 20 mg/L MA, then 40 mg/L MA, for 4 days each. Mice were weighed and the relative sides of the MA and non-MA cylinders were switched every 2 days, consistent with procedures used during selective breeding (Wheeler et al., 2009).

Table 4.2. Solutions offered for each experimental group and general timeline for Experiment 3.

	PHASE 1			PHASE 2	
Group	**Day -1 - 0**	**1-4**	**5-8**	**9-12**	**13-16**
Group 1: MA in NAC vs NAC	H2O/H2O	20MA in NAC/NAC	40MA in NAC/NAC	40MA in H2O/ H2O	40MA in H2O/ H2O
Group 2: MA in H2O vs H2O	H2O/H2O	20MA in H2O/H2O	40MA in H2O/H2O	40MA in H2O /H2O	40MA in H2O /H2O
Group 3: NAC vs NAC	H2O/H2O	NAC/NAC	NAC/NAC	20MA in H2O /H2O	40MA in H2O /H2O
Group 4: H2O vs H2O	H2O/H2O	H2O/H2O	H2O/H2O	20MA in H2O /H2O	40MA in H2O /H2O

Experiment 4: Glutamate-related protein expression

Seventy-two MA-naïve MADR mice were used (18/sex/line). Brains were extracted and placed in ice cold PBS, then cut into 5mm coronal sections. The NAcc core and shell were dissected out. Due to the small size of the NAcc core and shell, there was not enough usable tissue from a single animal to quantify protein. Thus, tissue from three animals were pooled into the same container, and this constituted a single sample. Only tissue from mice from the same cage was pooled. This yielded 24 total samples (6/sex/line). Samples were stored at -80°C.

Chilled lysis buffer was added to the frozen tissue. Samples were sonicated, centrifuged at 25,000 rpm for 20 min at 4°C, and supernatant extracted. Protein levels were assessed using a BCA assay. 10µg of protein from each sample was combined with sample buffer (1:10 XT sample buffer and XT reducing agent, BioRad ETC), boiled at 95°C, and stored at 4°C. Samples were loaded onto 12% Bis-Tris PrecastGel (Bio-Rad, Hercules, CA), then electrophoresed at 200V for 1-hour, then proteins were transferred to PVDF membranes for 1-hour at 100V. Blocking was done with 5% nonfat dry milk [source] in Tris-buffered saline and 1% Tween (TBST). Membranes were probed with the relevant antibody, washed in TBST for 5-min, before being probed with a secondary antibody (goat anti-mouse, 1:6000, Bio-rad, Hercules, CA). Proteins were visualized with enhanced chemofluorescence substrate (GE Healthcare, Piscataway, NJ). Protein densities were analyzed relative to β-actin using Image-Pro (version 6.3, Media Cybernetics, Silver Springs, MD). Each sample was run in triplicate, normalized per membrane, and averaged for each sample of pooled tissue. Samples were averaged per line and expression normalized to MALDR expression levels.

Statistical analysis

Data were analyzed using Statistica 13 Academic software (TIBCO Software Inc., Palo Alto, CA, USA). MA intake (mg/kg) and total volume (ml/kg) were analyzed with repeated-measures analysis of variance (ANOVA), with time or MA concentration as the repeated factor, and sex, dose or experimental group as possible independent variables. Significant interactions

were examined with simple main effects analyses, and Neuman–Keuls *post hoc* mean comparisons were performed when appropriate. Effects were considered significant at $p < 0.05$. Protein expression was analyzed with t-tests.

Results

Experiment 1: The effects of mGluR5 NAMs on established MA drinking

MTEP

Data for MTEP treatment are presented in Figure 4.1. A repeated measures ANOVA with a nested design analyzed the effects of injection period (vehicle injections and MTEP injections) MTEP dose, sex, and time (each 2-hour block) on 2-day average MA intake. There were no significant effects of sex or MTEP dose on MA intake. When data for the sexes and doses were combined, a significant time x injection period interaction was found ($F_{2, 92} = 3.20$, $p < 0.05$). When the effect of time was analyzed within each injection period, for both injection periods there was a significant increase in MA intake over time (Figure 4.1). When the effects of injection period were analyzed within each 2-hour block, during the last 2-hour block of the MTEP injection period, mice had significantly greater MA intake than during the last 2-hour block of the vehicle injection period ($p < 0.05$).

VU0424238

Data for VU0424238 treatment are presented in Figure 4.2. A repeated measures ANOVA with a nested design analyzed the effects of injection period (vehicle injections and VU0424238 injections) VU0424238 dose, sex, and time (each 2-hour block) on 2-day average MA intake. In the initial analysis there were no sex nor dose effects, so the analysis was performed with data for the sexes and doses combined. The significant main effect of time remained ($F_{2, 90} = 6.23$, $p < 0.001$), but there were no other effects.

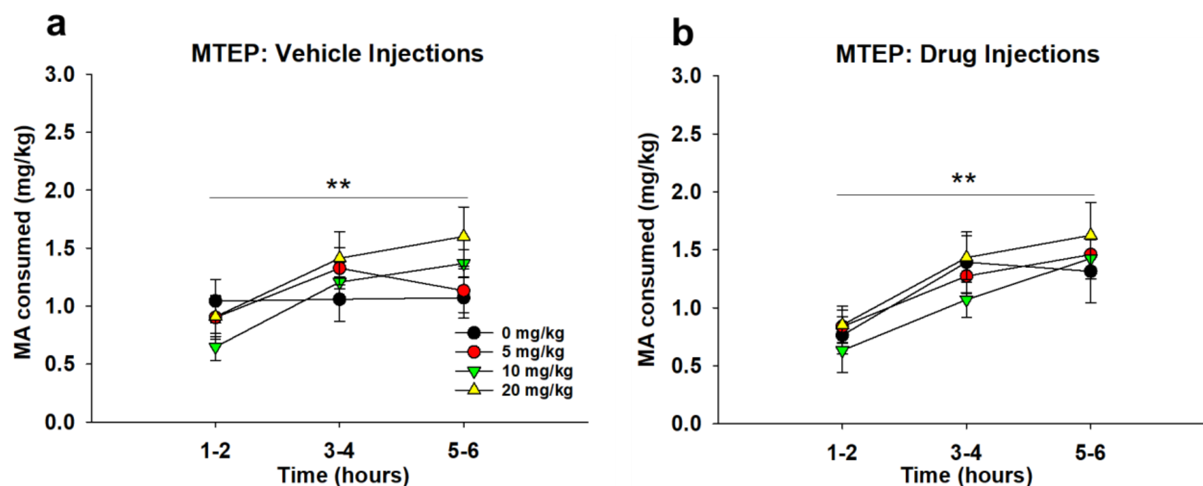

Figure 4.1. MTEP does not reduce established methamphetamine (MA) intake in mice bred for high MA intake. Because sex did not play a role in MA consumption, the data are collapsed across sex. Shown is MA consumption following (a) vehicle injections and (b) MTEP injections across the 3, 2-hour blocks during which mice had access to MA. Data are means ± SEM. **$p < 0.01$ for the simple main effect of time during that injection period. MTEP: 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine

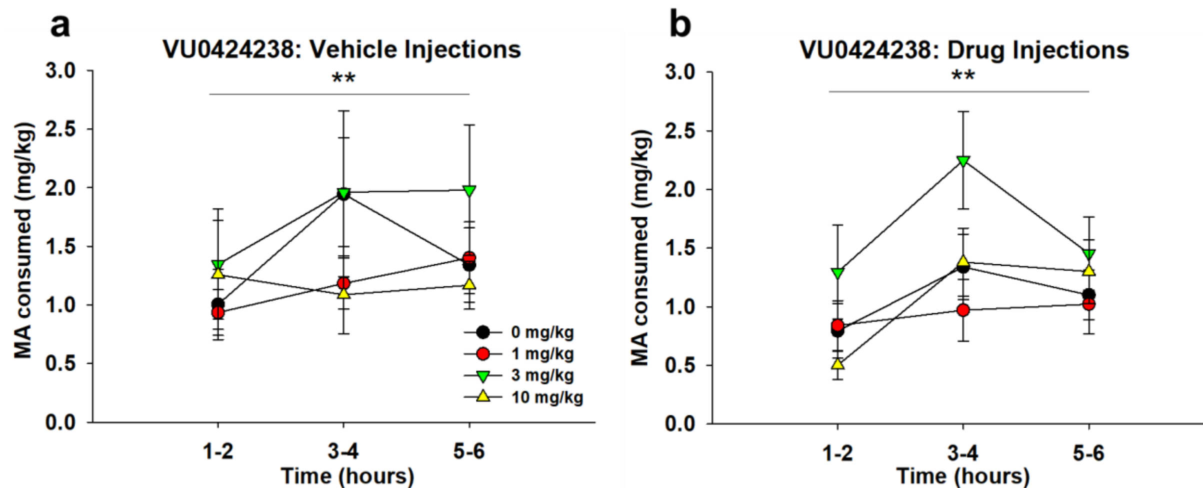

Figure 4.2. The mGluR5 negative allosteric modulator (NAM) VU0424238 does not reduce established methamphetamine (MA) intake in mice bred for high MA intake. Because sex did not play a role in MA consumption, the data are presented for the sexes combined. Shown is MA consumption following (a) vehicle injections and (b) VU0424238 injections across the 3, 2-hour blocks during which mice had access to MA. Data are means ± SEM. **$p < 0.01$ for the simple main effect of time during that injection period.

Experiment 2: The effects of an mGluR2 PAM, mGluR5 NAM, and xc⁻ modulator on the acquisition of MA drinking

Data for Experiment 2 are presented in Figure 4.3. Although all the drugs were tested in the same set of mice, analyses for each drug were performed separately. For each drug, a repeated measures ANOVA with a nested design analyzed the effects of the drug, sex, MA concentration, and time (each 2-hour block) on MA intake, measured as an average of days 2 and 4 of each MA concentration. These were the days mice were not weighed.

VU0424238

The initial analysis found a significant sex x dose (vehicle or VU0424238) interaction ($F_{1, 16} = 5.42$, $p < 0.05$). Simple main effects analyses found that during vehicle injections, male mice consumed slight, but significantly more MA (about 0.5 mg/kg) than female mice ($p < 0.05$). However, within each sex there was no significant change in intake between vehicle and drug injection periods. Thus, the analysis was run with data for the sexes combined. There was a significant MA concentration x treatment interaction ($F_{1, 18} = 5.38$, $p < 0.05$). Simple main effects analyses revealed that for the 20 mg/L MA concentration, there was no effect of VU0424238, and mice treated with VU0424238 actually increased MA intake during the 40 mg/L MA access period ($p < 0.05$). This was due to an increase in MA intake with increased MA concentration in mice treated with VU0424238, whereas the saline group did not increase MA intake over MA concentrations.

BINA

An initial analysis found no significant effects of sex so the analysis was conducted with the sexes combined. There was only a significant effect of MA concentration ($F_{1, 18} = 11.91$, $p < 0.01$). This was due to an increase in MA intake with increasing MA concentration ($p < 0.01$).

NAC

The initial analysis revealed a significant sex x treatment interaction ($F_{1, 16} = 5.64$, $p < 0.05$). Simple main effects analyses found that during vehicle injections, male mice consumed slight, but significantly more MA (about 0.5 mg/kg) than female mice ($p < 0.05$). However, within each sex there was no significant change in intake between vehicle and drug injection periods. Thus, the analysis was run with data for the sexes combined, and there remained only a significant effect of MA concentration ($F_{1, 18} = 8.37$, $p < 0.01$), which was due to an increase in MA intake with increasing MA concentration ($p < 0.05$).

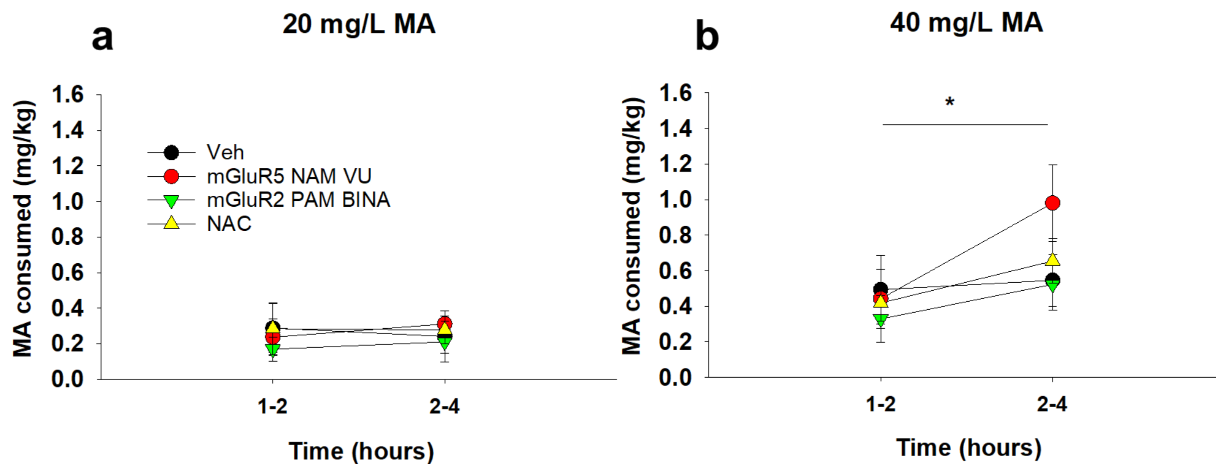

Figure 4.3. The mGluR5 negative allosteric modulator (NAM) VU0424238, the mGluR2 positive allosteric modulator Biphenylindanone A (BINA), and the cysteine prodrug n-acetylcysteine (NAC) do not inhibit the acquisition of methamphetamine (MA) intake in mice bred for high MA intake. Because sex did not play a role in MA consumption, the data are presented for the sexes combined. Shown is MA consumption during the (a) 20 mg/L and (b) 40 mg/L MA access periods across the 2, 2-hour blocks during which mice had access to MA. Data are means ± SEM. *$p < 0.05$ for the simple main effect of time in the VU0424238 treated group.

Experiment 3: The effects of oral NAC on MA intake

Groups 1 and 2 tested the hypothesis that NAC administered simultaneously with MA would inhibit MA intake. Groups 3 and 4 tested the hypotheses that pretreatment with oral self-administered NAC would attenuate MA intake. This resulted in groups 1 and 2 receiving access to MA for 8 days before groups 3 and 4 were ever presented MA (Table 4.2). It was only during the final 4 days of the study when all groups received the same MA solutions. This made comparisons of all groups inappropriate for most of the study. Because of this we analyzed data for groups 1 and 2 (co-administration of NAC and MA) and groups 3 and 4 (NAC pretreatment) separately. Then, a separate analysis was conducted on the final 4 days of the study to compare MA intake of all groups since they all had access to the same MA concentration at this time.

To test the hypothesis that NAC administered simultaneously with MA could inhibit the acquisition of MA drinking, a repeated measures ANOVA was conducted on data from Phase 1. MA concentration was the repeated measure, and it included sex and experimental group. Data are presented in Figure 4.4a. In the initial analysis there were no significant effects of sex. When the analysis was run with the sexes combined, there was only a significant effect of MA concentration ($F_{1, 16} = 16.25$, $p < 0.001$), due to an increase in MA consumption as the MA concentration increased ($p < 0.001$).

To determine if there were residual effects of NAC and MA co-administration, Phase 2 drinking data from groups 1 & 2 were analyzed (Figure 4.4b). A repeated measures ANOVA on data from Phase 2, with time (the 1st 4 days vs the 2nd 4 days) as the repeated measure. It included sex and treatment group. There were no effects of sex or treatment group, but there was a significant effect of time ($F_{1, 19} = 18.49$, $p < 0.001$). This was actually due to a decrease in MA intake over time ($p < 0.001$).

To test the hypothesis that pretreatment with orally administered NAC would inhibit the acquisition of MA drinking, data from Phase 2 was analyzed. Phase 1 was also analyzed for any

differences in total volume consumed. There were no differences in total volume consumed during Phase 1 (Figure 4.4c). Data for Phase 2 are presented in Figure 4.4d. A repeated measures ANOVA, with MA concentration was the repeated measure, and sex and experimental group as factors, found only a significant effect of MA concentration ($F_{1, 21} = 50.23$, $p < 0.001$). This was due to an increase in MA intake with increasing MA concentration ($p < 0.001$).

Finally, a factorial ANOVA compared average MA intake (mg/kg) of days 14 and 16 between all groups. For this analysis, whether mice had access to MA during Phase 1 (groups 1 and 2), and whether mice had access to NAC during Phase 1 (groups 1 and 3) were included as independent variables along with sex. There was a significant sex x MA history interaction ($F_{1, 24} = 4.39$, $p < 0.05$), however when history of NAC exposure was removed from the analysis this interaction was no longer present.

Experiment 4: Glutamate-related protein expression

Data are presented in Figure 4.5 There were no significant differences between the MADR lines in mGluR5, mGluR2, Homer2a/b, EAAT3, xCT, EAAT2, nor xCT expression in either the NAcc core or shell.

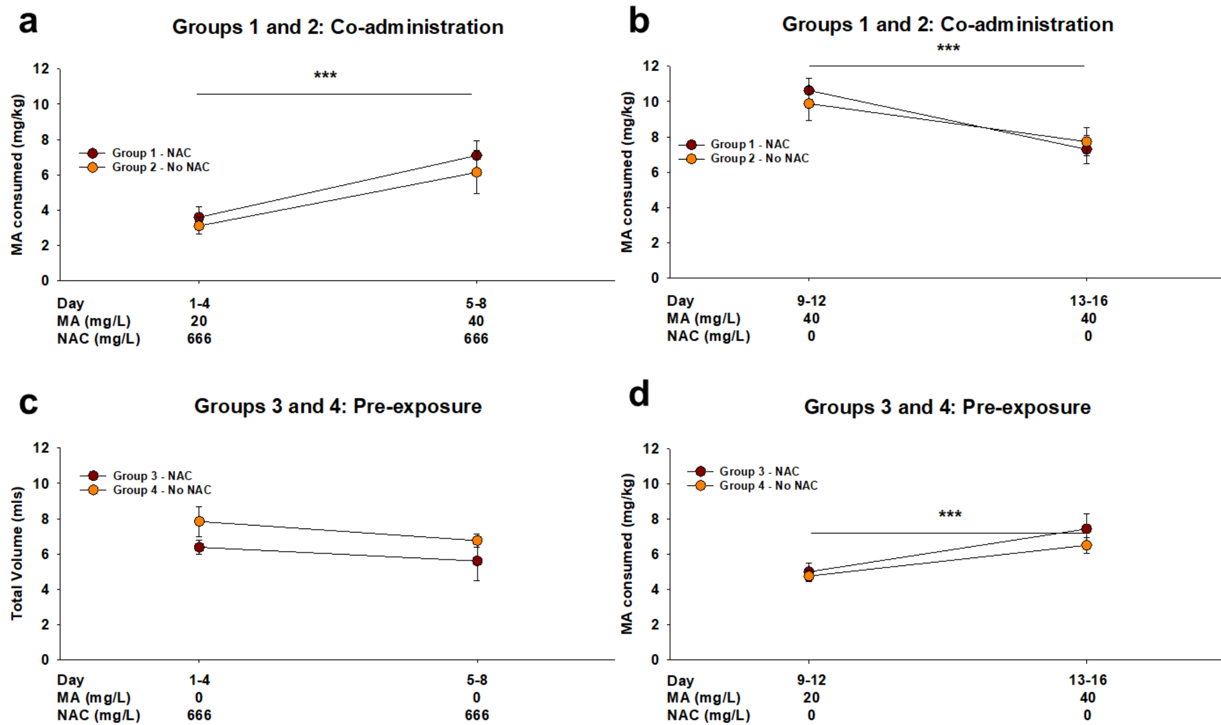

Figure 4.4. Oral intake of the cysteine prodrug n-acetylcysteine (NAC) does not inhibit the acquisition of methamphetamine (MA) intake in mice bred for high MA intake. Because sex did not play a role in MA consumption, the data are presented for the sexes combined. NAC was consumed either (a-b) during the acquisition of MA drinking, or (c-d) prior to the acquisition of MA intake. Data are means ± SEM. ***$p < 0.001$ for a change in MA intake across (a, d) MA concentrations and (b) across MA access days.

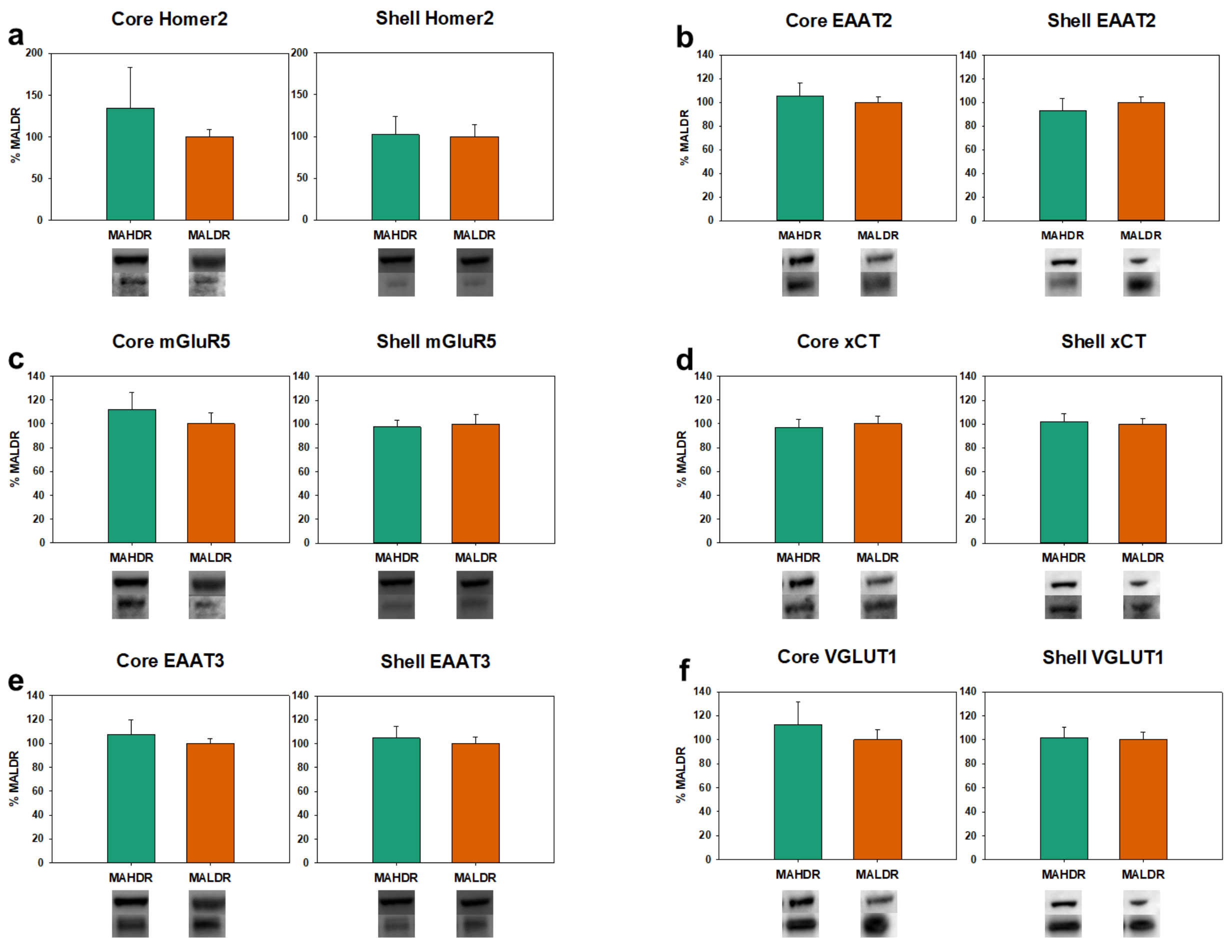

Figure 4.5. Western blot analysis of nucleus accumbens core and shell tissue for the expression of Homer2, EAAT2, mGluR5, xCT, EAAT3, and VGLUT1. Selective breeding for methamphetamine intake (MA) did not impact expression of any of the measured proteins. Data are shown as optical density as a percentage of MALDR line expression. MAHDR: MA high drinking line; MALDR: MA low drinking line; EAAT2: excitatory amino acid transporter 2; EAAT3: excitatory amino acid transporter 3; VGLUT1: vesicular glutamate transporter 1

Discussion

Our results suggest that glutamate dysregulation in the NAcc may not contribute to the high MA intake phenotype in the current replicate of the MAHDR line mice. None of the drugs tested were capable of reducing established or acquired MA intake in these mice. Additionally, there were no differences between the MADR lines in glutamate-related protein expression within the NAcc core or shell.

Disruption of the glutamate system appears to be a key characteristic of chronic drug exposure and may underlie relapse in some models (Kalivas, 2009; Quintero, 2013; Scofield et al., 2016). After repeated drug administration, x_c^- surface expression decreases, reducing extrasynaptic glutamate levels (Bowers et al., 2004; Ghasemzadeh et al., 2009; Kalivas, 2009; Kau et al., 2008; Knackstedt et al., 2009, 2010; Liechti et al., 2007; Madayag et al., 2007; Moussawi et al., 2009; Pendyam et al., 2009; Xi et al., 2002). Concurrently, PFC-NAcc synapses are potentiated, occluding LTP (Bowers et al., 2004; Ghasemzadeh et al., 2009; Liechti et al., 2007; Moussawi et al., 2009; Xi, 2002). This is likely due, in part, to decreased mGluR2 surface expression and decreased glutamatergic tone resulting from the loss of x_c^- (Grover & Yan, 1999; Kalivas, 2009; Moran et al., 2005; Wu et al., 2004; Zheng-Xiong Xi et al., 2002). Meanwhile, LTD is inhibited in part due to a downregulation of mGluR5 and reduced glutamatergic tone at remaining mGluR5s (Kasanetz et al., 2010; Lüscher & Huber, 2010). Finally, after reinstatement of drug-seeking, depotentiation occurs, which is also likely mGluR5-dependent (Jedynak et al., 2016; Kalivas, 2009; Kourrich, Rothwell, Klug, & Thomas, 2007; Kupchik et al., 2012; Martin et al., 2006; Thomas, Beurrier, Bonci, & Malenka, 2001). Targeting these changes using mGluR5 antagonists, mGluR2 agonists, and restoration of x_c^--derived glutamate, have all demonstrated efficacy in reducing drug self-administration, and cue- and drug-induced reinstatement of drug seeking (Kalivas, 2009).

The reduction in surface mGluR5 expression may be compensatory, as antagonists of mGluR5 attenuate both drug reinforcement and reinstatement, including for MA (Brown et al.,

2012; Gass et al., 2009; Herrold et al., 2013; Hodge et al., 2006; Niedzielska-Andres et al., 2021; Osborne & Olive, 2008; Palmatier et al., 2008; Tronci et al., 2010). An mGluR5 NAM was not able to attenuate MA intake in the studies presented here though. Blocking mGluR5 activity with the mGluR5 NAMs MTEP or VU0424238 did not reduce established MA drinking, and VU0424238 could not inhibit the acquisition of MA drinking. First, it is important to note that ours is not the first study to find mGluR5 antagonists to be ineffective at reducing drug-related behaviors (Palmatier et al., 2008; Roohi, Sarihi, Shahidi, Zarei, & Haghparast, 2014), alluding to additional mechanisms not yet understood. This is highlighted by our incomplete understanding as to why a seemingly compensatory reduction in LTD occurs in the first place. Previously, MAHDR line mice from the 2nd selection replicate of the 5 replicates produced so far, were found to have increased levels of NAcc mGluR5 (Szumlinski et al., 2017), but this difference was not found in the 5th replicate tested here. mGluR5 may have contributed to the earlier replicate's drinking phenotype, but not this replicate of MAHDR mice. It also may not be involved in the high drinking phenotype of MAHDR mice. Although *Taar1* genotype has proven to have a large influence on MA intake in the MAHDR lines, it does not account for all of the variance between MADR line mice. The MA drinking phenotype of MAHDR line mice is an aggregate of TAAR1-dependent and TAAR1-independent factors. Because of the overwhelming influence of *Taar1* genotype, any one of the additional factors contributing to high MA intake may be overshadowed. mGluR5 could therefor still be contributing to high MA intake in MAHDR line mice, but blocking it is insufficient to create detectible changes in MA intake.

Antagonizing mGluR5, activating mGluR2, and stimulating extrasynaptic glutamate release through x_c^- all failed to attenuate the acquisition of MA drinking when drugs were injected intraperitoneally. We had hypothesized that selective breeding for high MA intake resulted in disrupted glutamate systems in MAHDR line mice which predisposed them to greater MA drinking. Thus, whatever is driving the acquisition of MA drinking in this replicate MAHDR line may be independent of x_c^-, mGluR2, and mGluR5. This could be specific to this replicate, or

the development of MA drinking in MHADR line mice could be independent of these proteins across replicates. Additionally, as was described above, high MA intake in MAHDR line mice is a combination of *Taar1* genotype and other, unresolved factors. All these proteins could contribute to high MA intake, but the effects of *Taar1* genotype occlude any effects of targeting these proteins individually.

When we tested whether or not NAC affected MA intake when it was orally self-administered, NAC had no effect on the acquisition of MA intake regardless of whether it was administered during MA access or prior to access. NAC is orally bioavailable and has become a popular focus of study for its potential to treat drug addiction (Grant et al., 2010; McKetin et al., 2017; Mousavi et al., 2015). Its failure to alter MA drinking in our study may have been due to the pharmacokinetics of orally administered NAC. With the concentrations used, mice may not have been getting an adequate dose to affect intake. Another possibility that applies to both oral and intraperitoneal NAC administration is the inadvertent activation of mGluR5. Increased extracellular glutamate is well-documented after NAC administration, and it activates both mGluR2 and mGluR5 in the NAcc (Kalivas, 2009; Kupchik et al., 2012; Moran et al., 2005; Moussawi et al., 2009; Xi et al., 2002). Whereas mGluR5 antagonism inhibits MA self-administration and reinstatement of MA seeking (Brown et al., 2012; Gass et al., 2009; Herrold et al., 2013; Hodge et al., 2006; Niedzielska-Andres et al., 2021; Osborne & Olive, 2008; Palmatier et al., 2008; Tronci et al., 2010), mGluR5 agonists do the opposite (Kalivas, 2009; Moussawi et al., 2009). Co-administration of NAC and an mGluR5 antagonist could be even more effective (Kupchik et al., 2012).

We found no differences in the expression of glutamate-related proteins within the NAcc between the MADR lines. This could indicate that the previously observed differences are not responsible for the MA drinking phenotypes of MADR line mice (Szumlinski et al., 2017). It is also possible that such glutamate differences contributed to the earlier replicate's MA drinking

phenotypes but not the replicate tested here (Szumlinski et al., 2017), but as the earlier replicate has since been terminated it is impossible to know for certain.

While it is true that drugs targeting the glutamate system have been extensively studied for their potential to alter drug-related behaviors, relatively few studies have examined the effects of the pharmacological targets used here on MA self-administration, and to our knowledge no studies have tried to use these drugs to interfere with the acquisition of MA self-administration. Mechanisms promoting the acquisition and maintenance of MA self-administration may be distinct from those promoting self-administration of other addictive drugs. NAcc glutamate dysregulation is implicated in compulsive drug-related behaviors (Barnes et al., 2005; Everitt et al., 2008; Everitt & Robbins, 2005; Gardner, 2011; Kalivas, 2009; Lüscher et al., 2020; Robbins & Everitt, 2002; Swanson, 2000; Vanderschuren & Everitt, 2004; Wise & Koob, 2014; Yin & Knowlton, 2006), but not all drug self-administration or drug-seeking is compulsive (Lüscher et al., 2020; Singer, Fadanelli, Kawa, & Robinson, 2018; Vandaele & Ahmed, 2021). The lack of efficacy of these drugs to reduce MA drinking in MAHDR line mice implies that perhaps MA drinking in these mice is controlled, rather than compulsive. No studies have examined the MAHDR mice for compulsive-like MA intake.

The MAHDR line is also a unique model of MA intake. Common inbred strains of mice and rats are frequently used when testing NAC, and mGluR2/5 agonists and antagonists, but this is the first time a line selectively bred for high MA intake has been used. Considering the unique genetic makeup of the MADR line, the role of *Taar1* genotype cannot be ignored. The *Taar1*^{m1J} encodes a non-functional receptor (Harkness et al., 2015), but there is dominance for the B6-like *Taar1*, which encodes a functional receptor (Harkness et al., 2015). Thus, MALDR mice express a functional TAAR1 and MAHDR mice do not (Harkness et al., 2015). This is important because the studies examining NAC or mGluR5 agonists used either rats or B6 mice (Gass et al., 2009; Herrmann et al., 2018; Osborne & Olive, 2008). While *Taar1* variants may exist between rat strains (this has not been investigated), evidence suggests rat *Taar1* produces

a functional receptor capable of eliciting behavioral effects when activated by artificial ligands (Liu et al., 2020; Revel et al., 2011; Wainscott et al., 2007). Thus, studies investigating glutamate targets for amphetamine or MA treatment have used only animal models expressing functional TAAR1, whereas the model used here (MAHDR line mice) expresses a non-functional TAAR1. Given TAAR1's known regulation of glutamate release (Underhill et al., 2019, 2018) and involvement in hypoglutamatergic hyperlocomotion (Revel et al., 2011), drugs acting on the glutamate system may not induce the same effects in MAHDR line mice as they do in other animal models.

Glutamate within the NAcc is highlighted in drug addiction, and the drugs tested here were intended to attenuate MA intake by dampening NAcc glutamate. Their drugs were administered systemically however. Glutamate projections exist all over the central nervous system, and can regulate NAcc function both directly and indirectly (Britt et al., 2012; Kalivas, 2009; Morales & Margolis, 2017). PFC glutamate illustrates this nicely. MAHDR line mice have higher baseline glutamate in the PFC than MALDR line mice, but MALDR line mice have a greater PFC glutamatergic response to a challenge dose of MA whereas MAHDR line display a decrease in PFC glutamate (Lominac et al., 2016). Regulation of PFC glutamatergic projections is largely under the control of GABAergic interneurons which receive glutamatergic inputs from numerous brain regions (Britt et al., 2012; Kalivas, 2009; Morales & Margolis, 2017). Hypothetically, an increase in glutamate in the PFC during MA exposure could stimulate GABAergic interneurons, inhibiting glutamate projections from the PFC to the NAcc, explaining the fairly unchanged glutamate levels observed in the NAcc of MALDR line mice following MA injections, but the elevated NAcc glutamate levels observed in MAHDR line mice (Szumlinski et al., 2017). The drugs tested here could have decreased glutamate in other parts of the brain, but by doing so indirectly increased glutamate in the NAcc, negating any direct effects of the drugs on NAcc glutamate.

It is also important to note that the lack of differences in any of the glutamate-related proteins does not preclude differences in trafficking of these proteins. There may be differences in membrane expression or even function of these molecules, rendering the drugs tested less effective at reducing MA intake. For instance, if mGluR5 is overexpressed in cell membranes of MAHDR NAcc neurons, they could have higher sensitivity the x_c^--derived glutamate, obstructing any effects of mGluR2 activation. If this is the case, they may also require substantially higher doses of mGluR5 antagonists to produce effects.

There are several limitations to these studies. Foremost we do not know if the drugs had the intended effects on glutamate levels and neuronal activity. NAC injections and oral NAC administration may not have sufficiently increased extrasynaptic glutamate, and the mGluR2 and mGluR5 drugs may not have substantially altered synaptic potentiation, as would be expected (Conn & Pin, 1997; Grover & Yan, 1999; Jedynak et al., 2016; Kalivas, 2009; Kourrich et al., 2007; Kupchik et al., 2012; Malenka & Bear, 2004; Martin et al., 2006; Nicoletti et al., 2011; Thomas et al., 2001; Wu et al., 2004). While we have used this behavioral model to successfully measure changes in MA intake (Eastwood & Phillips, 2014a), the effects caused by these drugs may be more subtle, and these drinking procedures may not have been sensitive enough to detect more subtle drug effects. We also only tested one dose of NAC and BINA. We chose doses within the higher range of those tested in mice (Brown et al., 2012; Galici et al., 2006; Herrmann et al., 2018; Jin et al., 2010; Reichel et al., 2011). If these high doses were able to reduce MA drinking other doses would have been explored; it remains possible that other doses could have effects on MA intake.

There is ample evidence that genetic predisposition contributes to some drug addictions, including MA (Aoyama et al., 2006; Ehlers et al., 2011). The MAHDR line offers a unique model of such risks. The failure of glutamate-related drugs to alter MA intake in these mice suggests that pharmacologically targeting the glutamate system may be insufficient to treat MA addiction in genetically high-risk individuals. However, the variable results we obtained across two

replicates, along with positive results in other models, encourages caution in this interpretation. The results presented here also indicate that mGluR2 and mGluR5 are not meaningfully involved in the development nor the maintenance of MA drinking in MAHDR line mice. Future studies should examine these drugs' effects on reinstatement of MA seeking in this line. It would be worthwhile to test additional doses of NAC and BINA using the same MA drinking procedures used in these studies.

There are several ways future studies using oral NAC administration could be altered to potentially yield effects. Pretreatment of oral NAC could be continued into the MA self-administration period, or mice could receive 24-hour access to NAC, rather than just the 18-hour access given here. Gavage NAC administration would deliver a bolus of NAC, guaranteeing the desired dose. This risks confounding effects due to stress however.

If TAAR1 function is required for these drugs to have any impact on MA intake they will never have an effect in MAHDR line mice. Tests utilizing passive administration of MA could help determine if TAAR1 function is necessary for glutamate-related drugs to have effects. For example, since NAC attenuates MA locomotor sensitization (Herrmann et al., 2018), an MA sensitization protocol using NAC in the MADR lines could be performed. Both MADR lines experience locomotor sensitization to 0.5 and 2.0 mg/kg MA, but only the MAHDR line experiences sensitization to 4.0 mg/kg MA (Shabani et al., 2011). Since MAHDR line mice are functional *Taar1* knockouts, any differences in the effects of NAC on MA sensitization can assumed to be due to TAAR1. Therefore one would expect NAC to attenuate MA sensitization at lower MA doses (0.5 and 2.0 mg/kg) in MALDR line mice but not MAHDR line mice, We have also recently used CRISPR-Cas9 to perform a *Taar1* allele swap, creating MAHDR line mice that possess the B6 *Taar1* allele (Stafford et al., 2019). These mice display MA-related behaviors similar to MALDR line mice (Stafford et al., 2019). These mice offer an even better model for testing the role of TAAR1 in glutamate-dependent effects of MA.

In conclusion, attempting to correct glutamate dysregulation is not necessarily an effective approach to reduce MA intake. This may apply only to models of high MA intake risk, and might be specific to the route of MA administration (e.g. oral vs intravenous). Future studies should examine different doses of the drugs tested here, and could explore the interaction of TAAR1, glutamate regulation, and MA effects.

Chapter 5:

General Discussion

Goals and main findings

The MADR lines were developed to understand the genetic and biological mechanisms underlying MA addiction risk and protection against MA addiction. These lines significantly differ in MA intake. In 5 independent selection replicates, the MAHDR lines consume more MA relative to the MALDR line, which consumes virtually no MA. Data have been published for the first 3 replicate sets of lines (Shabani, McKinnon, Reed, Cunningham, & Phillips, 2011; Wheeler et al., 2009; Hitzemann et al., 2019). Figure 5.1 shows data for those 3 sets and replicates 4 and 5 (unpublished). The main goals of this dissertation were to investigate the mechanisms contributing to MA intake, from molecular to physiological, and to use the MAHDR line to explore potential pharmacotherapies.

Here, I characterize phenotypes important for MA intake ranging from molecular changes, to physiological responses to drugs, to behaviors arising from neurotransmitter system perturbations. The main findings are presented in Table 5.1. The findings include data on the efficacy of drugs targeting the glutamate system as potential pharmacotherapies to treat MA addiction. In Chapter 1, I report results from radioligand binding assays. These found *Taar1*^{m1J} possessed by D2 mice, and passed to MAHDR line mice, encodes a receptor (mTAAR1-D2) with drastically reduced binding capabilities. In Chapter 2, I present findings from a series of studies measuring thermal responses to several drugs in the MADR lines. A drug with a similar chemical structure to MA, MDMA, produced a pattern of temperature responses similar to MA, characterized by hypothermia in MALDR line mice, not observed in MAHDR line mice. Cocaine, a non-ALS stimulant, produced comparable hypothermia in both MADR lines. The 3rd drug tested, morphine, produced hypothermia in both lines, but MAHDR mice were significantly more sensitive to this effect. Morphine does not activate TAAR1, but MAHDR line mice consume less morphine than MALDR mice (Eastwood & Phillips, 2014a), suggesting some genetic component

tied to MA drinking selection intersects with avidity for opioids. Genotyping of the morphine treated mice revealed genetic linkage between *Oprm1* and *Taar1* such that MAHDR line mice are much more likely to possess the D2 *Oprm1* allele and MALDR line mice are more likely to possess the B6 *Oprm1* allele. Using BXD RI mice, I verified that it is *Oprm1* and not *Taar1* genotype driving sensitivity to morphine hypothermia. The final component of Chapter 2 tested the temperature altering effects of the partial OPRM1 agonist buprenorphine in MADR line mice. Buprenorphine induced hypothermia in both lines, but it was modest, and line-differences were confounded by sex effects. Overall, the hypothesis that buprenorphine attenuated MA intake in a previous replicate of MAHDR line mice (Eastwood & Phillips, 2014a) by inducing hypothermia was not supported. Chapter 3 presents data on the efficacy of pharmacologically targeting the glutamate system to alter MA intake in MAHDR line mice. Administration of the x_c^- prodrug NAC, two mGluR5 NAMs, and the mGluR2 PAM BINA, did not alter the acquisition nor the expression of MA intake. Furthermore, in the 5th selection replicate of the MADR lines, there were no observable differences in levels of Homer2a/b, EAAT2, EAAT3, xCT, or VGLUT1 in the NAcc.

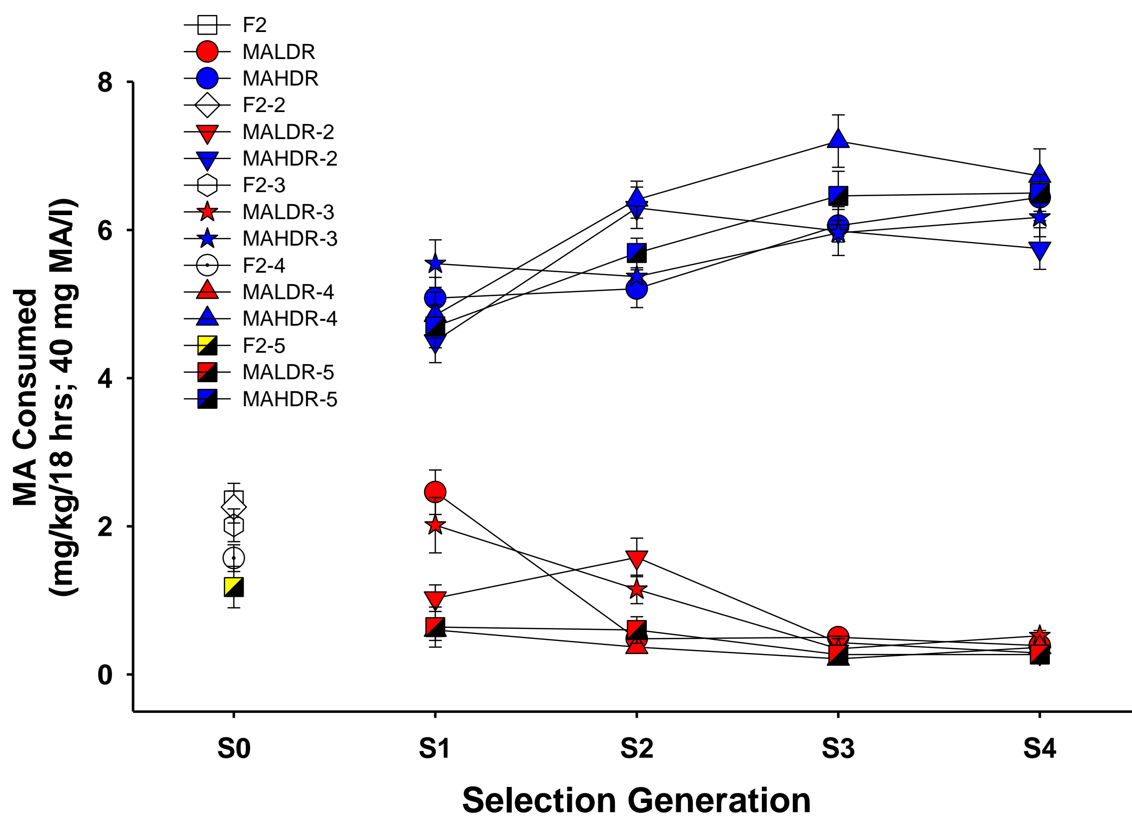

Figure 5.1. MAHDR line mice consume more MA during an 18 hour 2-bottle choice procedure, than MALDR line mice across all selection generations for all 5 selection replicates. MAHDR: methamphetamine high drinking; MALDR: methamphetamine low drinking

Table 5.1. Summary of results

Chapter	Model	Drug	Dose/Conc.	Test	Result
2	HEK-B6	$[^3H]$RO5166017	0.31-100 nM	Saturation binding	K_d = 0.516; B_{max} = 417.969
2	HEK-D2	$[^3H]$RO5166017	50-1000 nM	Saturation binding	K_d = 35.075; B_{max} = 3573.807
3	MAHDR/MALDR Rep 4/5	Cocaine	0, 15, 30 mg/kg	Thermal response	MAHDR = MALDR; hypothermia in both lines at all doses
3	MAHDR/MALDR Rep 4/5	MDMA	0, 2.5, 5 mg/kg	Thermal response	MAHDR > MALDR; hypothermia in only the MALDR line at all doses
3	MAHDR/MALDR Rep 4/5	Morphine	0, 15, 30 mg/kg	Thermal response	MAHDR < MALDR; hypothermia in both lines, but greater in MAHDR line
3	MAHDR/MALDR Rep 4/5	Morphine	-	*Taar1* & *Oprm1* genotyping	Linkage disequilibrium between the 2 genes; MAHDR line mice predominantly *Oprm1*-D2; MALDR mice predominantly *Oprm1*-B6
3	BXD RI strains	Morphine	0, 15, 30 mg/kg	Thermal response	*Oprm1*-B6 determines magnitude of thermal response to morphine

3	MAHDR/MALDR Rep 5	Buprenorphine	0, 1, 2, 4, 8, 16, 32 mg/kg	Thermal regulation	MAHDR = MALDR; hypothermia in both lines
3	MAHDR/MALDR Rep 5	Buprenorphine	-	*Taar1* and *Oprm1* genotyping	Linkage disequilibrium between the 2 genes; MAHDR line mice predominantly *Oprm1*-D2; MALDR mice predominantly *Oprm1*-B6
4	MAHDR Rep 5	MTEP (mGluR5 NAM)	0, 5, 10, or 20 mg/kg	Established MA drinking	No change in MA intake
4	MAHDR Rep 5	VU0424238 (mGluR5 NAM)	0, 1, 3, or 10 mg/kg	Established MA drinking	No change in MA intake
4	MAHDR Rep 5	VU0424238 (mGluR5 NAM)	0, 10 mg/kg	Acquisition of MA drinking	No change in MA intake
4	MAHDR/MALDR Rep 5	BINA (mGluR2 PAM	0, 32 mg/kg	Acquisition of MA drinking	No change in MA intake
4	MAHDR Rep 5	NAC	0, 100 mg/kg	Acquisition of MA drinking	No change in MA intake
4	MAHDR Rep 5	NAC	666 mg/L		No change in MA intake

4	MAHDR/MALDR Rep 5	-	-	Western blots	No differences in mGluR2, mGluR5, Homer2a/b, xCT, or VGLUT1 expression in the NAcc core/shell

TAAR1

Previous evidence indicated that the proline to threonine substitution found in *Taar1^{m1J}* eliminated receptor function. That research determined there was no cAMP response in the presence of ligand in cells expressing mTAAR1-D2 (Harkness, Shi, Janowsky, & Phillips, 2015; Shi et al., 2016). The reason for this was not clear; however, it was predicted that P77T altered the binding pocket enough to greatly reduce ligand binding. As discussed in Chapter 2, knowledge of proline's function in receptor dynamics and the position of the mutation, within the second transmembrane domain near a purported binding pocket stabilizing residue (Reese et al., 2014), made the conclusion that the receptor was nonfunctional reasonable. However, the discovery of an additional, cAMP-independent signaling pathway opened the possibility that mTAAR1-D2 was still capable of activity. Now we have direct evidence that binding is dramatically reduced for the mutant receptor. There was still some ligand binding. However, this binding is clearly insufficient to produce a physiologically relevant cAMP response at ligand concentrations that are pharmacologically relevant (Harkness et al., 2015; Shi et al., 2016), and is likely insufficient to engage any other signaling pathways as well. Using archived samples, we determined that *Taar1^{m1J}* arose between 2001-2003 in D2 mice from the Jackson Laboratory (Reed et al., 2017). Studies performed prior to this found no differences between B6 and D2 mice in temperature response to MA or amphetamine (Grisel et al., 1997; Seale, Carney, Johnson, & Rennert, 1985), nor do studies using D2 mice from vendors other than the Jackson Laboratory (Kita et al., 1998; Rabinak, Orsini, Zimmerman, & Maren, 2009).

In Chapter 1, I discuss the promise of TAAR1 as a target for pharmacotherapies. Previous research from our lab (Harkness et al., 2015) and the results I present in Chapter 2 on *Taar1^{m1J}*, highlight a need to consider genetic variation in animal research. Obviously spontaneous mutations like *Taar1^{m1J}* cannot be entirely accounted for before they are discovered, but special attention should be given to the genes encoding pertinent molecular targets when deciding on animal models and vendors. *Taar1^{m1J}* also emphasizes the

importance of tracking behavioral phenotypes. Considering the length colonies of inbred strains can be maintained, this is particularly important for these animal models. Literature reviews to confirm phenotypes have not shifted over time in published data is perhaps the minimum requirement, but verifying that the phenotype of interest has not changed through in-house experiments is a better practice when possible. There are documented variants in human *TAAR1* that reduce or eliminate receptor function (Shi et al., 2016). This stresses the need to understand individual variability, even for psychiatric diseases other than MA addiction, which I will discuss later.

Hypothermia and addictive drugs

Selective breeding for differential MA intake conferred differential sensitivity to the temperature altering effects of certain drugs. The chromosome 10 QTL accounted for > 60% of the genetic variance in MA intake in the MADR line (Belknap et al., 2013). Genetically correlated traits such as sensitivity or resistance to MA-induced hypothermia, are likely due to genes within this QTL, or genes regulated by genes in this QTL. *Taar1* was found to be a QTG for MA intake (Phillips et al., 2021; Stafford et al., 2019), and selective breeding resulted in $Taar1^{m1J}$ homozygosity in the MAHDR line (Harkness et al., 2015; Mootz et al., 2020). Because of the overwhelming effect of $Taar1^{m1J}$ on MA intake in the MAHDR line, it was reasonable to predict that genetically correlated traits were also mediated by $Taar1^{m1J}$. In Chapter 3 I report evidence that thermal response to both MDMA and morphine support this. MA is a TAAR1 agonist (Bunzow et al., 2001; Wolinsky et al., 2007) and can produce hypothermia and hyperthermia (Harkness et al., 2015; Matsumoto et al., 2014; Sprague et al., 2018). MDMA is likewise a TAAR1 agonist (Berry et al., 2017; Simmler et al., 2016), and in Chapter 3 I demonstrated that MDMA produces hypothermia in MALDR line mice but not MAHDR line mice, much in the same way MA produces hypothermia in MALDR line mice only (Harkness et al., 2015). It is highly probable that sensitivity to MDMA-induced hypothermia is determined by *Taar1* genotype. I expanded on this with a drug that does not act on TAAR1, morphine. Morphine produced

hypothermia in a dose and *Oprm1*-genotype dependent manner. I report genetic linkage between *Oprm1* and *Taar1.* Selective breeding resulted in segregation of *Taar1* genotypes in the MADR lines, and genetic linkage of *Oprm1* caused segregation of *Oprm1* genotypes as well. The consequences are enhanced sensitivity to the hypothermic effects of morphine in MAHDR line mice, and reduced sensitivity to these effects in MALDR line mice. The MADR lines' progenitor strains, the D2 and B6 inbred strains, show a similar relationship with hypothermia and morphine preference, such that D2 mice consume less morphine (Belknap, Crabbe, Riggan, et al., 1993; Doyle et al., 2008, 2014), and are more sensitive to the hypothermic effects of morphine than B6 mice (Belknap et al., 1989; Takamura Muraki & Ryuichi Kato, 1987).

As covered in Chapter 1, a connection between sensitivity to the hypothermic effect of a drug and drug intake is difficult to deny, at least for certain drugs. Where an association is found, there is an inverse relationship between magnitude of hypothermia and amount of drug intake, such that greater drug-induced hypothermia corresponds with lower drug intake. The cause of this is not clear; however, evidence suggests it is mediated by aversion. Some of this I covered in Chapter 1. To summarize, the MADR lines are a clear example of this. MALDR line mice develop hypothermia in response to MA whereas MAHDR line mice do not (Harkness et al., 2015). Accordingly, MAHDR mice are also substantially less sensitive to the aversive effects of MA than MALDR mice (Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). I discussed two hypotheses to explain this. Hypothermia may extend the period during which negative associations can be made (Christianson et al., 2005; Misanin et al., 2002, 1998); hypothermia itself could also be aversive.

It is possible that the interaction of hypothermia and drug intake is entirely coincidental. That is, hypothermia could be a byproduct of central nervous system effects of certain drugs, but the relative rewarding or aversive effects of that drug are not contingent on or modified by hypothermia. Studies showing that altering ambient temperature can affect conditioned aversion argue against this. Raising ambient temperature reduces ethanol and LiCl-induced CTA and

CPA (Cunningham et al., 1988; Cunningham & Niehus, 1993). Two sets of studies that raised ambient temperatures during MA, MDMA, and cocaine self-administration in male Wistar rats support the hypothesis that hypothermia extends the period during which negative associations can be formed (Cornish et al., 2008, 2003). When rats were trained to self-administer intravenous MDMA (0.1, 0.3, 1.0 mg/kg) under an FR-1 schedule in a 21 °C room, raising the room temperature to 30 °C increased the number of infusions for all doses (Cornish et al., 2003). Under the same conditions, rats also increased their responding for a 1 mg/kg dose of cocaine (Cornish et al., 2003). Raising the ambient temperature also increased self-adminsitration of a 0.1 mg/kg MA infusion under an FR-1 schedule and progressive ratio schedule (Cornish et al., 2008). Higher ambient temperatures did not increase self-administration of a D1 receptor agonist either (Cornish et al., 2008). In all experiments warmer room temepratures actually decreased locomotor activity (Cornish et al., 2008, 2003), rulling out increased overall activity as an explanation. Nor did increased ambient temperature alter MA metabolism (Cornish et al., 2008). The studies with MDMA and cocaine did not measure body temperatures, so the possibility that the rats experienced drug-induced hypothermia in the colder rooms cannot be excluded. Body temperatures were measured following MA self-administration though. There was a 1-2 °C increase in body temperature following MA self-administation in the colder and the warmer room (Cornish et al., 2008). Taken together, these studies indicate that warmer core body temperatures increase the rewarding value of a drug. If hypothermia increases the period during which negative associations can be made, then perhaps hyperthermia decreases that period. There were a few aspects of the studies that make interpretation difficult. All studies used within-subjects designs. The increases in body temperature following MA self-administration could have been due to some factor (perhaps locomotor activity) during the self-administration session. Also, a bolus IP injection of 3 mg/kg MA only increased body temperature 0.5 °C in the 21 °C room, and only after 2 hours post-injection. Temperatures were not measured between 30 min and 2 hours post-injection, so how

hyperthermia progressed is not known. Considering the entire self-administration sessions were 2 hours long, and rats self-administered < 0.1 mg/kg MA during that period, it is not likely that the hyperthermia reported after MA self-administration was solely due to MA. The exact cause of the hyperthermia does not necessarily matter though, as the hyperthermia itself could still be decreasing the time window in which aversive conditioning could occur.

The hypothesis that hypothermia is aversive has not been thoroughly studied, but knowledge of mammalian thermoregulation also lends its support. Proper thermal regulation is necessary for survival. Every species has a "thermoneutral zone" which it works to maintain (Cannon & Nedergaard, 2011; Ganeshan & Chawla, 2017; Morrison & Nakamura, 2019; Terrien, Perret, & Aujard, 2011), and see taxon-specific responses developed to maintain this thermoneutral zone. Some are autonomic, like shivering (Cannon & Nedergaard, 2011; Crawshaw, 1980; Crawshaw, Grahn, Wollmuth, & Simpson, 1985; Ganeshan & Chawla, 2017; Mota-Rojas et al., 2021; Tansey & Johnson, 2015). Others take the form of goal-oriented behaviors, such as huddling or moving to a warmer area (Almeida, Steiner, Branco, & Romanovsky, 2006; Crawshaw, 1980; Crawshaw et al., 1985). Both behavioral and autonomic thermoregulation are controlled by the hypothalamus, but studies manipulating the temperature of the hypothalamus indicate that it is easier to elicit the behavioral responses than the autonomic responses (Morrison & Nakamura, 2019; Mota-Rojas et al., 2021; Refinetti & Carlisle, 1986; Terrien et al., 2011). Disruptions to thermal regulation are a substantial threat to fitness, and stressful to rodents (Bańka et al., 2013; Okuda et al., 1986; Shida et al., 2020; Terrien et al., 2011). Consequently, the dirsuptions to body temperature by certain drugs could be enough to motivate rodents to avoid a drug or drug-paired cues.

Misanin et al. (1998) tested the effects of hypothermia on aversion learning, and found that hypothermia alone could not produce CTA (Misanin et al., 1998). However, they induced hypothermia by immersing rats in cold water, which is an external produced threat to the rodent's thermoneutral zone. This is in contrast to hypothermia produced by opioids, MA, and

ethanol. We cannot know how this hypothermia is perceived to rodent subjects, but it is clearly drastically different than hypothermia produced by cold water immersion. Additionally, rats in a cold environment can be conditioned to press a lever to turn on a heat lamp (Weiss & Laties, 1961). Thus, escape from a cold ambient temperature can be used as a reward to reinforce behavior.

All of the drug-induced hypothermia data to date do not distinguish between hypothermia itself being aversive and an increase in aversive conditioning due to hypothermia. One way to address this would be to target cold-sensing neurons directly. The type 8 transient receptor potential ion channel (TRPM8) is expressed in cold sensing neurons (Mota-Rojas et al., 2021; Tan & Knight, 2018). Selective ablation of TRPM8-expressing neurons or knockout of TRPM8 virtually eliminates some thermoregulatory behaviors in mice (Knowlton et al., 2013) and greatly reduces the temperature required for a mouse to voluntarily leave a cold surface (Knowlton et al., 2013). TRPM8 antagonists induce hypothermia, and block warmth-seeking behaviors in rodents (Camila Almeida et al., 2012; Gavva et al., 2012). Ablation of TRPM8-expressing neurons or TRPM8 antagonists can be used to inhibit the ability to sense cold in mice. If these manipulations also attenuated drug aversion, or increased intake of drugs typically avoided (such as MA in the MALDR mouse line), that would be evidence for drug-induced hypothermia itself being aversive. This would not dissociate aversion to hypothermia from enhanced aversive conditioning entirely, but would go a long way toward that end.

Opioids and the opioid system

Because most recreational and clinically used opioids act on OPRM1, substantial research has gone into understanding the functional variants of OPRM1 and how they relate to addiction risk. In humans a common SNP, A118G, is associated with greater risk of opioid and alcohol addiction (Bond et al., 1998; Kreek, Bart, Lilly, Laforge, & Nielsen, 2005; Tan, Tan, Karupathivan, & Yap, 2003). For example in one study, in Indian and Hispanic populations, the A118G is more prevalent in individuals with opioid addictions (Bond et al., 1998; Tan et al.,

2003). Efforts to understand the functional consequences of this polymorphism have found that A11G results in lower OPRM1 expression (Kroslak et al., 2007) and a receptor with lower binding affinity (Beyer, Koch, Schröder, Schulz, & Höllt, 2004). Human participants have similar physiological response to morphine, as measured by pupil size, regardless of whether or not they possessed A118G (Lötsch et al., 2002). However, participants homozygous for A118G required nearly twice the dose of an active metabolite of morphine, morphine-6-glucuronide, to produce physiological effects as participants who did not possess A118G (Lötsch et al., 2002). Another study found that women who possessed at least 1 copy of A118G had reduced musculoskeletal pain following a motor vehicle collision, but in men A118G increased musculoskeletal pain (Linnstaedt et al., 2015). Considering the research suggesting OPRM1 variants affect addiction risk for some ethnicities and not others (Bond et al., 1998; Kreek et al., 2005; Tan et al., 2003). It seems that in humans, a number of genetic factors mediate OPRM1-related risk for opioid addiction.

In Chapter 1, I touched on Mop2, a QTL for oral morphine intake in the B6 and D2 strains (Berrettini et al., 1994; Doyle et al., 2008; Ferraro et al., 2005). This 7.6 Mb QTL contains *Oprm1* (Doyle et al., 2014). While other candidate genes do lie within this QTL, efforts to identify the QTG in this region have largely focused on *Oprm1*. Several SNPs were identified in the *Oprm1* promotor of B6 and D2 mice that result in a modest increase in activity of the D2 promotor, but this only translated to a trend toward increased OPRM1 mRNA in the forebrain of D2 mice (Doyle et al., 2006). When radiolabeled [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin ([^{3}H]DAMGO) was used to measure OPRM1 in B6 and D2 mice, no differences in receptor density nor binding affinity in the NAcc, mPFC, or ventral midbrain were found (Eastwood et al., 2018). In contrast, MALDR line mice expresses significantly more OPRM1 in the PFC than MALDR line mice (Eastwood et al., 2018). MAHDR line mice actually express similar PFC ORPM1 density to B6 and D2 mice (Eastwood et al., 2018). *Oprm1* is regulated by a gene

expression network associated with risk for MA intake in the MADR lines (Belknap et al., 2013), which mostly likely explains the OPRM1 expression differences between the MADR lines.

The OPRM1 expression results our lab found (Eastwood et al., 2018) partially contradict previous work that found greater OPRM1 density in the frontal cortex of D2 mice, relative to B6 mice (Petruzzi, Ferraro, Kürschner, Golden, & Berrettini, 1997). Petruzzi et al. (1997) did acknowledge that there was substantial variability in measurements of D2 OPRM1 density and this finding requires more investigation (Petruzzi et al., 1997). They also used the entire frontal cortex, whereas we dissected out only the mPFC and used a tissue punch. The difference in precision could explain the discrepancy in OPRM1 densities. Our lab only measured baseline OPRM1 density, but Petruzzi et al. (1997) measured OPRM1 density following repeated morphine treatment as well. Repeated morphine exposure did not alter OPRM1 density or binding in the frontal cortex, but there was a significant decrease (approximately halved) in OPRM1 density in the striatum of B6 mice due to morphine treatment (Petruzzi et al., 1997). Genetic drift also remains a possibility. Between the times these earlier studies were performed and when our lab measured OPRM1 density, *Taar1*^{m1J} arose in the D2 strain (Reed et al., 2017). Other unrecognized genetic changes may have occurred that modify *Oprm1* expression.

Despite inconclusive evidence that *Oprm1* is the QTG for morphine intake in the Mop2 QTL, it remains a primary candidate (Doyle et al., 2008, 2014). However, the refined Mop2 QTL encompasses at least 22 genes (Doyle et al., 2008, 2014). Of the genes investigated other than *Oprm1*, 4 are differentially expressed between B6 and D2 mice, or within-strain expression is modified by opioid exposure (Doyle et al., 2008, 2014; Liu et al., 2002). The regulator of G-protein signaling 17 gene (*Rgs17*) lies within Mop2 and is one of the stronger candidate genes aside from *Oprm1*. *Rgs17* forms a complex with OPRM1 and is involved in the long-term desensitization of OPRM1 following morphine exposure (Garzón, Rodríguez-Muñoz, López-Fando, & Sánchez-Blázquez, 2005b, 2005a; Rodríguez-Muñoz, Bermúdez, Sánchez-Blázquez, & Garzón, 2007; Rodríguez-Muñoz, de la Torre-Madrid, Sánchez-Blázquez, & Garzón, 2007). In

D2 mice, greater *Rgs17* mRNA was found in the frontal cortex, NAcc, and midbrain/brainstem relative to B6 mice, and more *Rgs17* protein expression was found in the midbrain/brainstem and NAC of D2 mice (Doyle et al., 2014). After morphine exposure, *Rgs17* protein expression in the midbrain/brainstem of D2 mice decreased, which was not observed in B6 mice (Doyle et al., 2014). The other 3 genes do not have the same clear connection as *Rgs17*. Iodotyrosine dehalogenase 1 (*Dehal1*) is more densely expressed in the midbrain of D2 mice relative to B6 mice (Doyle et al., 2014), but is also upregulated in B6 mice implanted with morphine pelets (Doyle et al., 2014). Other than this not much is known about the interaction of *Dehal1* and opioids. Another gene, proteinphosphatase 1 regulatory inhibitor subunit 14c (*Ppp1r14c*), is upregulated in the hippocampus and thalamus of B6 mice following acute and repeated morphine exposure (Liu et al., 2002), and *Ppp1r14c*-KO mice show blunted morphine CPP (Drgonova, Zimonjic, Hall, & Uhl, 2010). The vasoactive intestinal polypeptide gene (*Vip*) is upregulated in the mPFC and midbrain of B6 mice implanted with a morphine pellet (Doyle et al., 2014). The product, VIP, induces analgesia (Mácsai, Szabó, & Telegdy, 1998), and the endogenous opioid met-enkephalin inhibits VIP release (Eklund, Sjöqvist, Fahrenkrug, Jodal, & Lundgren, 1988). None of these genes were identified in the gene expression network MA drinking risk (Belknap et al., 2013). Other genes for morphine intake lying within Mop2 are also part of the gene expression network (Belknap et al., 2013; Doyle et al., 2008, 2014). For instance, protein-L-isoaspartate [D-aspartate] O-methyltransferase 1 (*Pcmt1*) was identified in the network and is within the Mop2 QTL, but has not been considered a QTG candidate for morphine drinking in the B6 and D2 strains, and has never been studied for a role in opioid or MA addiction, to the best of my knowledge.

Whether the QTG for morphine encompassed in the Mop2 QTL is *Oprm1* or not, there is good reason to believe that genes within Mop2 impact morphine-induced hypothermia. Seeing as *Oprm1* is genetically linked to *Taar1*, other genes in Mop2 may also be linked to *Taar1*. I did

not genotype the mice used in Chapter 3 for anything but *Taar1* and *Oprm1*, but existing MADR line mice could be genotyped for the other genes within the Mop2 QTL.

One and 2 mg/kg buprenorphine successfully attenuated the acquisition of MA intake in MAHDR line mice (Eastwood & Phillips, 2014a). When I tested 1-32 mg/kg buprenorphine for thermal effects in the MADR lines, the results did necessarily support hypothermia as a mechanism by which buprenorphine could limit MA intake. The hypothermia induced by buprenorphine completely dissipated by 90 minutes post-injection. At doses capable of decreasing MA intake, buprenorphine produced only modest hypothermia, and potential sex effects make it unlikely that buprenorphine-induced hypothermia is the primary mechanism by which it limited MAHDR MA intake (Eastwood & Phillips, 2014a). The duration of hypothermia induced by buprenorphine in Chapter 3 is far shorter than one would expect. In male B6 NMRI mice the half-life of a 0.05 mg/kg subcutaneous injection was 3.7 hours (Kalliokosk et al., 2011), and in female CD-1 mice, the half-life of an intravenous 2.4 subcutaneous buprenorphine injection is about 3 hours (Yu et al., 2006). Subcutaneous injections of 1.5 mg/kg buprenorphine produce analgesia, hyperlocomotion, and suppress respiration for at least 4 hours in male Swiss-Webster mice (Healy et al., 2014). In female Wistar Hannover rats, subcutaneous doses of 0.03 and 0.15 mg/kg buprenorphine increased body temperature, locomotor activity, and heart rate (Ilbäck, Siller, & Stålhandske, 2008). The effects on body temperature and heart rate persisted significantly longer than the hypothermic effects I report in Chapter 3, upwards of 6 hours (Ilbäck et al., 2008), compared to the 90 min maximum duration I reported. It is unlikely that this discrepancy between the duration of hypothermic responses I presented and pharmacodynamic and pharmacokinetic effects reported elsewhere is due exclusively to the animal model used, considering the difference in effect duration is a matter of hours. Despite the inconsistent temperature altering effects of buprenorphine I report here, the potential for buprenorphine as a treatment for MA and opioid addiction warrants further investigation.

Glutamate

In Chapter 4, I report that in the 5th selection replicate of the MADR lines, there are no differences in levels of Homer2a/b, EAAT2, EAAT3, xCT, or VGLUT1 in the NAcc core or shell. This could be because these proteins, and perhaps the glutamate-system as a whole, is not connected to the MA drinking phenotypes of the MADR lines regardless of replicate. In this case, the previously observed differences in glutamate-related proteins and glutamate levels were coincidental. Another possibility is that in the earlier replicate of MADR line mice tested, which were from the 2nd selection replicate, glutamate was important for the MA-intake phenotype, but is not for this most recent replicate. In the 3rd selection replicate, an analysis assessing how selection impacts the relationship between genes implicated a number of glutamate-related genes, including *Homer2* (Hitzemann et al., 2019), which was found to be upregulated in the MAHDR line mice from the 2nd replicate (Szumlinski et al., 2017). This argues that glutamate is important for the differential drinking phenotypes of at least 2 selection replicates.

We can confidently say that *Taar1* is the largest driver of MA intake differences between all replicates of MADR lines, but it is not the sole driver of their MA drinking. Other than *Taar1*, different genetic factors may contribute to each replicate of MADR lines MA drinking phenotypes, which would explain the discrepancies in glutamate protein levels between different replicates.

In the 2nd selection replicate, MAHDR line mice had higher baseline glutamate in the NAcc, and a greater increase in glutamate following a challenge dose of MA, relative to MALDR line mice (Szumlinski et al., 2017). Whether the protein level differences found in this replicate were related to the differences in extracellular glutamate is not known. In the earlier replicate of MADR lines EAAT3 levels were lower in the NAcc MAHDR line mice (Szumlinski et al., 2017), but EAAT3 is not the predominant regulator of extracellular glutamate in the central nervous system (Bjørn-Yoshimoto & Underhill, 2016; Nieoullon et al., 2006). Xc^- was never measured in

this earlier replicate, but differences in Xc^- expression or function could have explained extracellular glutamate differences.

The decision to target the specific glutamate receptors and transporters I did in Chapter 4 was informed by existing literature supporting these targets for pharmacotherapies. The decision was also predicated on earlier research in the MADR lines demonstrating line-dependent differences in the levels of some of these proteins and in extracellular NAcc glutamate (Szumlinski et al., 2017). If glutamate dysregulation contributed to previous replicates' MA drinking phenotypes but not the current replicate, that could explain why the drugs I tested failed to alter MA intake. Another major consideration is the impact of *Taar1*. Considering swapping *Taar1*^{m1J} for the reference allele can reverse the MA drinking phenotype of MAHDR line mice (Stafford et al., 2019), the presence of a non-functional TAAR1 may be so strong that certain pharmacological interventions cannot alter MA intake. Duration of action of these drugs is also a concern. I tried to limit this problem by limiting MA access periods to 4-6 hours after drug injections. Allowing mice to freely consume NAC over for 18 hours was also partially motivated by this same concern.

As was detailed in Chapter 4, NAC increases extrasynaptic glutamate, which will necessarily activate mGluR2 and mGluR5. Activation of mGluR2 reduces drug intake, but mGluR5 activation promotes it. The ability of NAC to decrease MA intake relies on the assumption that mGluR2 activation will outweigh the effects of mGluR5 activation. This might not have been the case for the studies I present in Chapter 4. An mGluR5 NAM may have to be administered to inhibit mGluR5 activation in order to see any effects on MA intake.

The glutamate system may also prove to be a beneficial target to treat MA neurotoxicity, Xc^- in particular. Xc^-—knockout mice are substantially less sensitive to 6-hydroxydopamine-induced neurotoxicity (Massie et al., 2011). Knockout of xCT or administration of an xCT inhibitor, both of which eliminate Xc^- function, reduced MA-induced glutamate release, oxidative stress, and dopaminergic loss in rat striatum (Dang et al., 2017).

Translatability to clinical applications

In the United States, genetic counseling dates back to the late 1960s, but has more recently seen applications for psychiatric diseases (Abacan et al., 2019; Moldovan, Pintea, & Austin, 2017). Understanding the functional consequences of variations in relevant genes will become increasingly important as this treatment approach expands. The increased interest in TAAR1 as a therapeutic target for psychiatric diseases (Dodd et al., 2021; M. D. Schwartz et al., 2018) expedites the need to better understand variants of this receptor. We know there are functional human TAAR1 variants (Shi et al., 2016), but the full consequences of these SNPs have yet to be elucidated. The results presented in this dissertation, and the relevant research characterizing phenotypes related to *Taar1*, promote a better understanding of how TAAR1 functions and how drugs, therapeutic and addictive, interact with it. This is vital to the clinical application of pharmacotherapeutics targeting TAAR1. This is true not just for MA addiction treatment. TAAR1 is a promising target for schizophrenia treatments (Revel, Moreau, et al., 2012; Revel et al., 2013; Schwartz et al., 2018), but these will not work if a patient possesses a non-functional TAAR1.

Unless thermal response to drugs is entirely disconnected from drug preference, there is clinical relevance in understanding drug-induced temperature changes. Temperature response can at the very least be a biomarker of risk, as long as correlations between body temperature changes and drug preference hold in humans. Understanding the common mechanisms of hypothermia and drug aversion may present novel therapeutic targets. Seeing as the hypothermic effects of MA are mediated by TAAR1 activation (Harkness et al., 2015; Stafford et al., 2019), continued research into TAAR1 may identify the common mechanisms of aversive effects of drugs and hypothermia. The hypothalamus is a logical starting place. The hypothalamus is central to thermal regulation (Morrison & Nakamura, 2011; Tan & Knight, 2018). Hypothalamic afferents to the VTA are activated by MA injections in B6 and Sprague-Dawley rats (Colussi-Mas, Geisler, Zimmer, Zahm, & Bérod, 2007; Tomita et al., 2013). *Taar1* is

also expressed in the hypothalamus (Borowsky et al., 2001; Lindemann et al., 2008), and it could mediate thermal responses to MA through activity there. In mice, *Taar1* is expressed in the preoptic area of the hypothalamus (Lindemann et al., 2008), a region known as vital for proper thermoregulation (Morrison & Nakamura, 2011; Tan & Knight, 2018). 3-Iodothyronamine (T1AM), an endogenous amine related to thyroid hormone involved in metabolism and temperature regulation (Scanlan et al., 2004), is a TAAR1 agonist and produces hypothermia in B6 mice (Gachkar et al., 2017; Panas et al., 2010; Scanlan et al., 2004). T1AM-induced hypothermia is significantly attenuated in *Taar1*$^{-/-}$ mice (Panas et al., 2010). T1AM also activates neurons in the hypothalamus of male B6 mice (Gachkar et al., 2017). These data hint at a central role of thermoregulation of TAAR1. Regardless of whether hypothermia alters aversive conditioning, or is aversive itself, TAAR1 activation within the hypothalamus could be the source of MA-induced hypothermia and consequently MA aversion.

We still do not know how TAAR1 activation mediates aversive effects of MA. Even if hypothermia is an important component of the aversive effects of MA, it is unlikely to be the sole effect driving aversion in the MALDR line, or any mouse model for that matter. Yet the presence of a functional TAAR1 results in profound aversion to MA, even when other genetic factors potentially promoting MA intake are present, as is the case with our *Taar1* allele-swapped MAHDR mice (Phillips et al., 2021; Stafford et al., 2019). Clarifying the mechanisms by which TAAR1 activation mediates aversion could present druggable targets for MA addiction treatment. As I have already discussed, TAAR1 regulates monoaminergic activity (Bradaia et al., 2009; Revel et al., 2011; Rutigliano et al., 2018; Xie & Miller, 2008; Xie & Miller, 2009a; Xie et al., 2007). The dorsal raphe nucleus (DRN) is the largest source of serotonergic neurons in the brain (Jacobs & Azmitia, 1992), and is important for encoding rewarding stimuli (Li et al., 2016). Stimulation of DRN serotonin neurons is also rewarding. Transgenic mice will nose-poke for optogenetic stimulation of DRN-VTA serotonin neurons, and optogenetic stimulation of these neurons produces CPP (Nagai et al., 2020). The DRN also expresses *Taar1* (Borowsky et al.,

2001; Lindemann et al., 2008; Xie & Miller, 2008). TAAR1 activation by endogenous or synthetic agonists decrease DRN serotonin neuron firing (Revel et al., 2011). Thus, TAAR1 activation by MA may inhibit DRN serotonin neuron activity in MALDR line mice, blocking some rewarding effects or promoting anhedonia.

Connecting differences in mouse *Taar1* and *Oprm1* to functional differences in human receptors can serve to identify high and low addiction risk individuals. This has benefits for treatment and prevention aside from predicting efficacy of pharmacotherapeutics. Knowledge of one's own addiction risk could be used to inform personal decisions on drug use. Knowledge of major genetic risks for addiction could also inform the prescription of drugs with high abuse potential. For instance, a doctor may limit or rule out opioids to manage pain in patients with high genetic risk for opioid addiction, and a psychiatrist may take extra precautions when prescribing Adderall to a patient with high genetic risk for MA and amphetamine addiction. This is the basis of individualized medicine, and has been applied to other diseases. For example, metabolism of the immunosuppressants mercaptopurine and azathiopurine are affected by functional variants of thiopurine S-methyltransferase (TPMT) (Evans et al., 2001; Evans & Relling, 2004; Evans, Horner, Chu, Kalwinsky, & Roberts, 1991; Weinshilboum, 2003). Some patients have variants of *TPMT* that render them TPMT deficient, and require significantly lower doses of mercaptopurine and azathiopurine (Evans et al., 2001; Evans & Relling, 2004; Evans et al., 1991; Weinshilboum, 2003). In fact, typical doses are toxic in these patients (Evans et al., 2001; Evans et al., 1991; Weinshilboum, 2003). Genotyping for *TPMT* can prevent this. Certain polymorphisms of the beta-adrenergic receptor result in hyper-functionality, and patients with these polymorphisms require higher doses of heart failure medication when being treated for hypertension (Taylor & Bristow, 2004; Terra et al., 2005)

I was not able to alter MA intake of MAHDR line mice by targeting the glutamate system, but this may be an indicator that the MAHDR line is a useful screening tool for pharmacotherapies. MAHDR line mice may represent a model of especially intractable MA

addiction. Buprenorphine was able to reduce MA intake in MAHDR line mice (Eastwood & Phillips, 2014a). This would signal buprenorphine is a pharmacotherapy worth testing in clinical trials, specifically for those patients who have been resistant to treatment [some buprenorphine clinical data]. Of course, buprenorphine could be acting on a mechanism driving MA intake unique to the MAHDR line. If this is the case such a mechanism has yet to be identified. Including MAHDR line mice or mice similarly prone to high intake of a drug of interest could help establish the limits of potential pharmacotherapeutics in preclinical trials. If a drug cannot alter drug-related behaviors in high drug intake risk animal models, then perhaps it is not suitable for those with severe drug addiction.

Summary of findings and future directions

In summary, the differences in MA intake between the MADR lines are predominantly due to the possession of a mutant *Taar1*, *Taar1^{m1J}*, by the MAHDR lines. In this dissertation I report that *Taar1^{m1J}* encodes a receptor with substantially reduced binding ability. I also report genetic linkage between *Taar1* and *Oprm1* in the MADR selected lines. Using the MADR lines and BXD RI strains, I confirm this has resulted in enhanced sensitivity to the hypothermic effects of morphine in the MAHDR line. The observed pattern of hypothermia to morphine aligns with a larger body of literature suggesting a negative correlation between drug preference and hypothermic response to that drug. Additionally, I tested drugs targeting the glutamate system, all of which failed to alter MA intake in MAHDR line mice. Finally, I found no differences in the levels of glutamate-related proteins within the NAcc of MADR line mice. This disagrees with previous finding (Szumlinski et al., 2017), indicating that factors other than *Taar1* are not necessary for the MA drinking phenotypes of any of the produced replicates.

An important future direction will be to characterize potential constitutive activity of the TAAR1 encoded by *Taar1^{m1J}*. Considering the role TAAR1 plays in transporter trafficking (Underhill et al., 2020, 2019), measuring membrane bound transporters from harvested tissue would be ideal. Tissue from *Taar1* allele-swapped MAHDR and D2 mice would dissociate the

effects of *Taar1*^{m1J} from other genetic effects that could influence transporter trafficking. Determining the exact effects of TAAR1 activation that are aversive and produce hypothermia is another important next step. As I explained, TAAR1 activation engages at least 2 independent signaling pathways (Underhill et al., 2020, 2019), resulting in rapid internalization of transporters which is terminated after approximately 15 minutes. Which of these pathways is responsible for aversive effects of TAAR1 activation will help reveal the causes of aversion to MA. Recall that TAAR1 activation initiates RhoA activation and subsequent internalization DAT and NET, which is halted by a parallel TAAR1-signaling pathway involving PKA (Underhill et al., 2020, 2019). A KO mouse that selective deletes an isoform of a catalytic subunit of PKA, Cβ1 (*Prkacb[β1]*$^{-/-}$), could be used to resolve the contributions of these 2 pathways to the effects of MA (Kirschner, Yin, Jones, & Mahoney, 2009). *Prkacb[β1]*$^{-/-}$ mice show no overt behavioral or physiological differences from WT littermate (Kirschner et al., 2009). Presumably, TAAR1 agonists like MA should cause greater DAT and NET internalization in these mice than WT mice, because the pathway halting transporter internalization should not be engaged in *Prkacb[β1]*$^{-/-}$ mice. This could be confirmed using the same methods as Underhill et al (2019). Brain tissue collected from *Prkacb[β1]*$^{-/-}$ mice and WT mice would be treated with amphetamine or MA and activated RhoA, and membrane-bound DAT and NET measured. Initial behavioral studies could compare MA self-administration, MA CPP.CPA, and/or MA CTA in these mice compared to WT littermates. As I mentioned in Chapter 2, Rho-kinase inhibitors could be used in MALDR line mice. Rho-kinases are downstream effectors of RhoA associated with amphetamine-induced DAT function (Inan & Büyükafşar, 2008; Wheeler et al., 2015). Simply injecting a Rho-kinase inhibitor before MA injections during an MA CTA or CPA procedure and measuring changes in acquired CTA and CPA compared to non-inhibitor controls would be viable.

It may be, and is in fact likely, that TAAR1-mediated aversive effects of MA depend on brain region. Selective knock-down of *Taar1* in specific brain regions could be performed. Short-hairpin RNA (shRNA), a method of RNA interference, offers one method to achieve this. This

method uses plasmid, viral, or bacterial delivery of artificial RNA that promotes enzymatic degradation of target mRNA, downregulating a gene of interest (Moore, Guthrie, Huang, & Taxman, 2010). shRNA knock-down of *Taar1* different brain regions, such as the hypothalamus or DRN, could be performed in MALDR line mice or B6 mice, and subsequent MA-induced CTA/CPA assessed for instance.

MA-induced hypothermia is markedly associated with MA aversion (Harkness et al., 2015; Shabani et al., 2011; Shabani, McKinnon, et al., 2012; Wheeler et al., 2009). We have yet to determine why though. A viable experiment would be something akin to the experiments that raised ambient temperatures to alter drug-related behaviors (Cornish et al., 2008, 2003; Cunningham et al., 1988; Cunningham & Niehus, 1993). Using implanted transponders (Miner, O'Callaghan, et al., 2017) to confirm core body temperature changes, we could perform CTA/CPA experiments in much the same way using MAHDR and MALDR line mice. If raising the ambient temperature of the room is able to prevent or attenuate MA-induced hypothermia in MALDR line mice, and subsequent CTA/CPA is also blocked or attenuated, this would confirm that internal body temperature is directly related to MA CTA/CPA. We could also reduce the ambient temperature of the room to try and produce hypothermia in MAHDR line mice. If reducing the ambient temperature can produce hypothermia in MAHDR line mice, and they begin to show MA CTA/CPA at MA doses that previously did not produce CTA/CPA, this would be a strong indicator that hypothermia is directly related to MA aversion. In Chapter 1 I specified that such experiments cannot determine why hypothermia contributes to aversion, which remains the case here. The use of TRPM8 agonists or antagonists could be used to determine the role of hypothermia in drug aversion. Antagonists co-administered with a drug of interest would diminish a subjects' ability to perceive cold. An increase in intake would verify that the perception of hypothermia is limiting intake. Similarly, TRPM8 agonists could be co-administered with a drug of interest. In this case a decrease in intake would signify perceiving hypothermia contributes to drug aversion. TRPM8 could also be selectively ablated in the

hypothalamus of rodents, and subsequent drug preference and/or sensitivity to aversive effects of a drug could be measured. The functional differences between the D2 and B6 OPRM1 need to be characterized. This has been attempted (Doyle et al., 2006; Eastwood et al., 2018; Petruzzi et al., 1997), but any causal link between observed polymorphisms, receptor levels, and receptor trafficking remain unknown.

Finally, other neurotransmitter systems could be targeted in the MADR lines. MAHDR mice have reduced basal DA in the NAcc and mPFC relative to MALDR line mice (Lominac et al., 2014). In response to a challenge dose of MA, MAHDR line mice also displayed an increase in mPFC DA not observed in the MALDR line (Lominac et al., 2014). Additionally MAHDR line mice have higher DAT levels in the NAcc than MALDR line mice (Lominac et al., 2014). In light of TAAR1-dependent regulation of DAT (Underhill et al., 2019, 2020; Xie & Miller, 2007, 2008; Xie & Miller, 2009a), there may also be differences in DAT trafficking between the MADR lines. This could be performed by measuring biotinylated DAT in tissue from the MADR lines.

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