Title

The effect of arrestin-3 engagement at the mu-opioid receptor and individual predispositions on the development of compulsive drug-seeking behavior in mice

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The effect of arrestin-3 engagement at the mu-opioid receptor and individual predispositions on the development of compulsive drug-seeking behavior in mice

By

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DISSERTATION

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Dissertation abstract

In 2020, 254 people died because of opioid overdose every day in the United States. Opioid analgesics are still regarded as the gold standard for alleviating pain clinically but present significant risks. Their analgesic utility (the effect) is limited by multiple side effects, including respiratory depression, constipation, and the development of an Opioid Use Disorder (OUD). A lack of consensus on the cellular signaling events responsible for these negative side effects remains a major limitation to the development of an opioid with a reduced effect/side effect profile.

All opioids exert their analgesic effects through the activation of the Gi-coupled μ- Opioid receptor (MOR). Acute action at MOR results in the reduction of neuronal excitability, reducing pain signal transmission. However, chronic use of opioid analgesics results in cellular adaptations. A central theory, biased agonism, seeks to explain the manifestation of these adaptations by comparison to endogenous signaling patterns. When MOR is activated by its endogenous ligand, a regulatory molecule called Arrestin-3 (Arr3) is recruited to the receptor, causing titration of signal transduction and subsequent internalization and recycling of the receptor. Morphine and other clinically used therapeutics do not effectively recruit Arr3. The lack of Arr3 recruitment results in receptors remaining present on the membrane without being internalized and recycled back to the membrane. Therefore, recruitment of Arr3 could be beneficial in preventing counter adaptations to chronic morphine. However, a competing theory states that Arr3 may mediate the negative side effects seen with continued opioid use. There is no consensus on the role of Arr3 in mitigating or causing the negative side effects seen in chronic opioid use.

To investigate the effect of Arr3 on drug-seeking behavior, this study utilizes three different genotypes of mice with varying Arr3 recruitment profiles in response to morphine: 1) WT(poorly recruits Arr3), 2) RMOR (strongly recruits Arr3), 3) Arr3

-KO (does not recruit Arr3). Chapter 2 utilizes a novel oral operant self-administration paradigm for 17 weeks to model the transition from impulsive to compulsive drug use, difficulties in ceasing drug-seeking behavior, and relapse. This model shows that 38% of animals in WT and Arr3-KO genotypes exhibit compulsive drug-seeking behavior, but 0% of RMOR mice become compulsive drug-seekers.

In Chapters 3-4, I investigate what underlying environmental causes explain why a subset of genetically homologous mice develop compulsive drug-seeking behavior. About 10-30% of persons prescribed opioids will develop an OUD. The explanation for why some individuals transition to misuse of opioids, placing themselves at higher risk for overdose, remains unclear. To model how individual behavior can predispose a subject to transition to compulsive drug use, a battery of anxiety measures was collected pre- and post-study to determine if baseline behavior or changes in behavior in response to drug exposure predict compulsive drug use. I determine that individual anxiety state does not influence compulsive drug-seeking behavior. In Chapter 5, I determine how changes in gut microbiome composition in response to morphine vary among individuals and how we can leverage these individual differences to develop microbial biomarkers of compulsive drug-seeking behavior.

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Chapter 1

Introduction

Introduction

I) Opioid epidemic prevalence and history

The opioid epidemic is the most severe public health crisis the United States of America has faced. The epidemic's toll has included 500,000 opioid-related deaths and an estimated $700 billion annually in economic burden [1]. This epidemic has had five phases. The first phase of the opioid epidemic was marked by a rise in prescription-related overdoses attributed to the FDA approval of Purdue Pharma's higher-dose, long release formulation of Oxycontin [2]. This drug was inadequately described as having less addictive potential and the number of prescriptions rose from 670,0000 to 6.2 million from 1997-2002 [3]. Concurrently, the American Pain Society advocated for pain to be counted as the fifth vital sign, causing clinicians to overprescribe opioids to manage pain [4]. The second phase of the opioid epidemic began as tightened regulation of opioid prescriptions precipitated rises in heroin overdoses. Approximately 3-5% of prescription drug users report using heroin in the same year [5]. Driven by declining heroin prices and scarcity of prescriptions, the lifetime prevalence of heroin use increased from .33 to 1.6% between 2001 and 2013 [6]. In 2013 the third phase opens with fentanyl, a synthetic opioid 50-100x more potent than morphine [7]. Overdose deaths from fentanyl and other synthetic opioids increased tenfold from 2010-2017, accounting for almost 60% of opioid-related deaths [8]. Emerging evidence now points to a fourth phase, associated with increasing fatalities from the combination of opioids with psychostimulants, such as cocaine and methamphetamines. This phase was worsened by the COVID-19 pandemic [9]. Previous promising trends projecting reduced overdoses were reversed, instead showing an increase of 28.5% from April 2020-2021. This sharp increase is attributed to several factors: 1) reductions in pain

management procedures and access to healthcare during pandemic-related closures, 2) CDC guidelines for forced tapering of opioid use, and 3) the marked additional stress of COVID-19 lockdowns and economic instability on mental health [9].

In addition to the increased risk of overdose, other negative side effects include respiratory depression, the development of tolerance to the analgesic effects, physical dependence on opioids, constipation, gut dysbiosis, and the development of an Opioid Use Disorder (OUD) [10]. The Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criteria defining an OUD are listed in Table 1 [11]. Briefly, an OUD is defined as having two or more of the following symptoms in a one-year period: difficulty limiting intake and craving, spending an excessive amount of time obtaining opioids, issues fulfilling work and social obligations, development of tolerance, and signs of withdrawal upon cessation. Clinical observation of these symptoms are well characterized, but the translation of acute molecular signaling events induced by opioids to the manifestation of these criteria lacks consensus. Despite these negative side effects, opioid analgesics remain the gold standard to treat serious pain without any effective alternatives. A clear need to develop new opioid pharmaceuticals that alleviate pain (effect) but limit these risks (side effects) is present. The search for an opioid with an improved effect/side effect profile remains a key goal.

Diagnostic Criteria:
Opioids are taken in a larger amount or longer than intended
Unsuccessful efforts to control opioid use
A great deal of time is spent obtaining, using, or recovering from opioids
Recurrent use resulting in failure to complete life obligations
Use despite persistent interpersonal problems causes by the effects of opioids
Abandonment of social, occupational, or recreational activities
Recurrent use in situations that are physically hazardous
Continued use despite knowledge of having a recurrent physical or psychological problem attributed to opioid use
Tolerance, defined as a need for markedly increased amounts to achieve intoxication or pain relief
Experiencing opioid withdrawal syndrome symptoms upon cessation including: anxiety, insomnia, dilated pupils, body aches, sweating, shaking, rapid breathing, hallucinations, and seizures

Table 1- Diagnostic criteria of opioid use disorder from the Diagnostic and Statistical Manual of Mental Disorders, Version 5. (DSM-V)

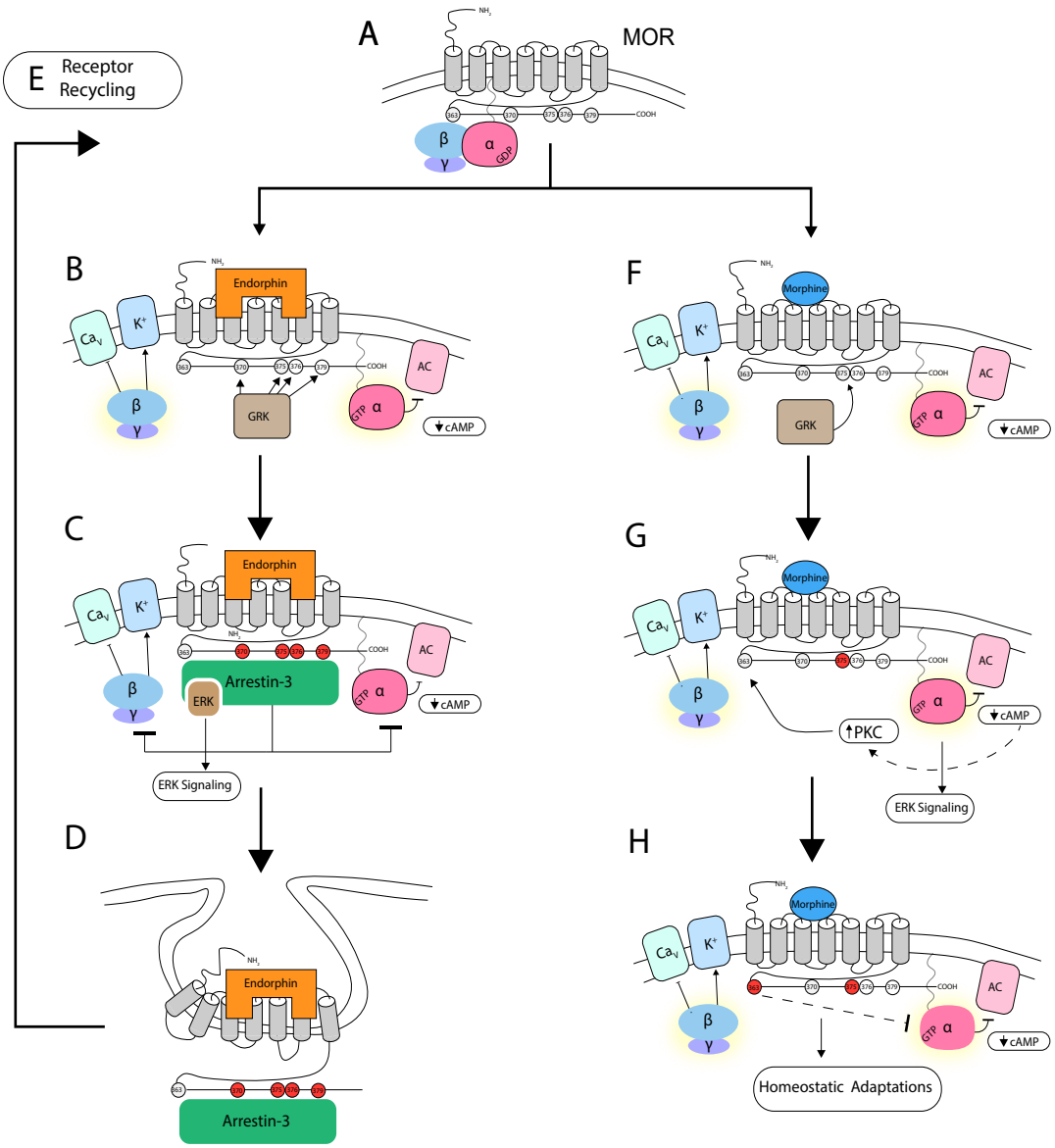

Figure 1.1- Mechanism of signal transduction at MOR in response to morphine and endorphin. A) MOR, a GiPCR sits in a resting state on a cell membrane. B) Endorphin binds to MOR and causes dissociation of the trimeric G-Protein (α,βγ) to cause inhibition of Adenylate Cyclase (AC) and voltage gated calcium channels (Ca_v), as well activation of potassium channels (K+). G-protein Kinase (GRK) phosphorylates residues T370, S375, T376, and T379. C) Arresin-3 is recruited to phosphorylated MOR. ERK signaling is activated. D) Arrestin-3 serves as a scaffold for protein to cause endocytosis of MOR. E) MOR is recycled back to the plasma membrane, devoid of ligand or phosphorylation. F) When MOR is activated by morphine, the same acute signaling events occur but GRK phosphorylates S375. G) Further desensitization of MOR by PKC occurs because of chronically reduced cAMP levels. H) Chronic morphine results in homeostatic adaptations to counteract lower cAMP levels. Morphine does not drive endocytosis.

II) Acute and chronic mechanisms of action at the Mu Opioid Receptor

Opioids exert their acute effects by binding the Mu-Opioid Receptor (MOR), an inhibitory G-Protein Coupled Receptor (G_iPCR; Fig. 1.1A). The endogenous ligands for MOR, endorphin, and enkephalins, bind and induce a conformational change that exchanges GDP for GTP. This causes the dissociation of the G-protein into alpha and beta-gamma subunits. The Gα effectively inhibits Adenylate Cyclase (AC), reducing cAMP. Lower cellular cAMP concentrations result in increasing conductance of K^+ channels to hyperpolarize the cell, reducing spontaneous excitability and inhibiting neurotransmitter release. The Gβγ subunits inhibit Ca^{2+} channels and GIRK currents, also reducing synaptic transmission (Fig. 1.1B).

Following the dissociation of the G-protein, the C-terminus of the MOR is phosphorylated by the G-protein receptor kinase (GRK) at the 'phosphorylation barcode' [12]. The MOR C-terminus barcode is comprised of 11 potential Serine/Threonine residues from T354-T394. Phosphorylation-specific antibodies and site-directed mutagenesis were used to identify several residues that regulate the recruitment of arr3: T370, S375, T376, and T379 [13–15]. Phosphorylation of these residues occurs sequentially: first at S375, followed by T370, then T376 and T379 (Fig. 1.1C) [13,16]. Complete phosphorylation of the barcode promotes efficient recruitment of arrestin-3 (Arr3) to the receptor. Recruitment of Arr3 halts signal transduction by sterically uncoupling the receptor from the G-protein and serves as a scaffolding protein to shuttle the desensitized receptor to a clathrin-coated pit (Fig. 1.1D). The receptor is then endocytosed, deliganded, dephosphorylated, and recycled back to the plasma membrane to await ligand binding once again (Fig. 1.1E) [17–19]. In contrast to the signaling evoked by endorphins, activation of the MOR by many clinically relevant opioids produces a different response. Here I will focus on morphine. Although the initial G-protein signal is identical (Fig. 1.1F), morphine-activated receptors are phosphorylated by GRKs at S375, but not at subsequent phosphorylation sites (T370,

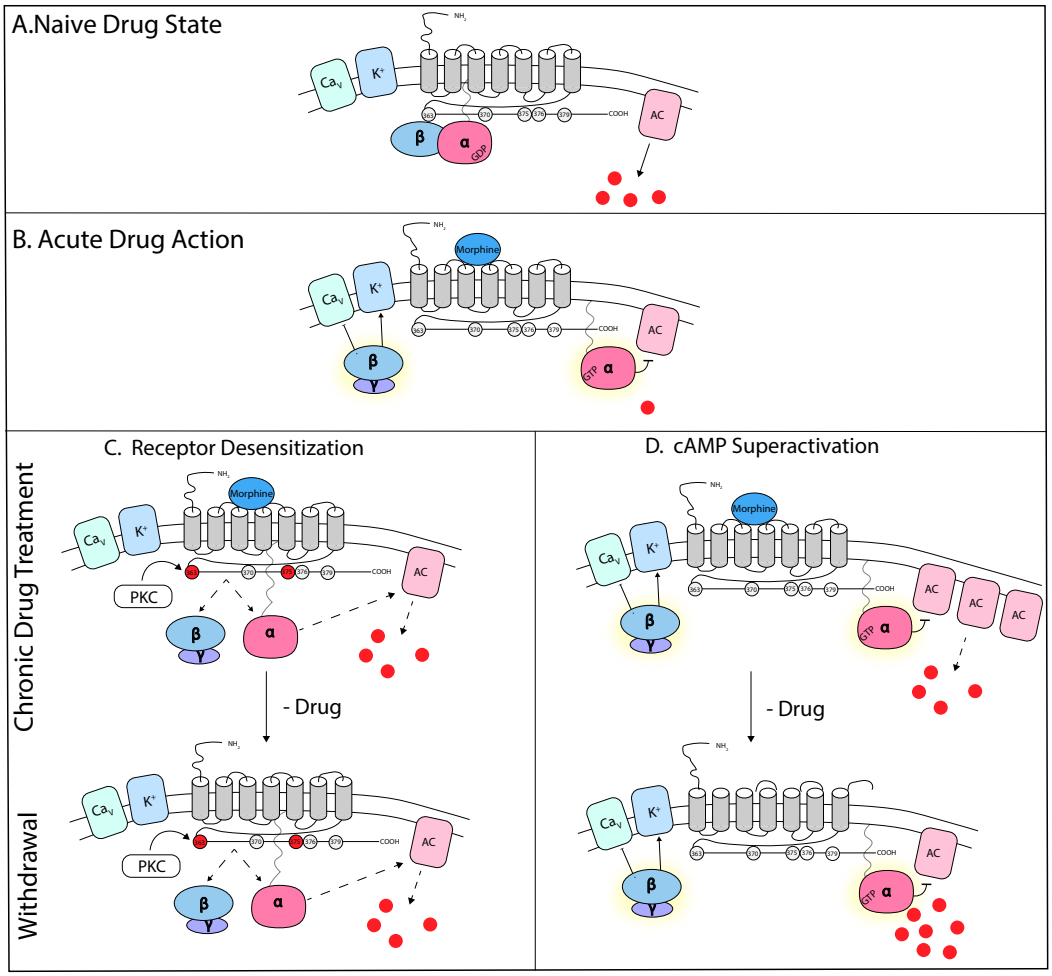

Figure 1.2- Adaptations to chronic morphine include receptor desensitization and cAMP superactivation. A) MOR in a naïve drug state. B) Acute drug action reduces cAMP and neuronal firing through decreased calcium and increased potassium conductance. C) MOR receptor desensitization by PKC and GRK results in an increase in cAMP (top, chronic drug treatment) compared to acute effect (B) but is not affected by the removal of morphine during withdrawal (bottom, withdrawal). D) Increased expression of AC results in an increase in cAMP (top, chronic drug treatment) compared to acute effect (B) and results in cAMP superactivation upon removal of morphine (bottom, withdrawal).

T376, and T379) [13,14] (Fig. 1.1G). This single phosphorylation event is not sufficient to promote efficient recruitment of Arr3 to the receptor and in turn, no endocytosis or receptor recycling occurs (Fig. 1.1H) [20,21]. The lack of endocytosis results in continuous cellular signaling, such as the continued repression of cAMP production.

Chronic opioid administration of morphine results in several cellular mechanisms

to counteract continuous cellular signaling. The binding of Arr3 to arrest the signal and induce endocytosis of the receptor is one such mechanism. However, morphine does not cause robust enough phosphorylation of the barcode region to recruit Arr3. Therefore, mechanisms to counteract the continued signaling from MOR are employed. Desensitization of MOR through partial phosphorylation is one such mechanism. Protein Kinase C (PKC) phosphorylation of MOR at S363 has been demonstrated by protein mass spectrometry and site-directed mutagenesis (Fig. 1.2C) [22,23]. Phosphorylation at S363 hinders G-protein association as shown by GTPγS assays. This hindered protein association desensitizes the receptor and reduces signaling, counteracting the Gi signaling from the chronic opioid stimulus (Fig. 1.2C) [23,24].

Alterations in gene expression are another counteradaptation mechanism employed by the cell to counteract chronic signaling. Activation of MEK/ERK by Gβγ regulates transcription factors like CREB and c-fos [25,26]. Gene targets include increased expression or activity of AC, upregulation of G_s-GPCR, and downregulation of other G_iGPCRs. These changes in gene expression result in hyperactive cAMP production. Previously physiological levels of cAMP are now achieved in the presence of morphine, despite morphine inducing Gi signaling (See Fig. 1.2A versus Fig. 1.2D, top). Compared to acute action (Fig. 1.2B), the presence of morphine in chronic drug treatment no longer reduces cAMP levels below physiological norms. In fact, the levels of cAMP in the presence of chronic morphine are similar to the levels in a naïve drug state (Fig. 1.1A versus Fig. 1.2D). This phenomenon is known as cAMP superactivation [27–29]. The removal of morphine causes previously repressed cAMP levels to increase rapidly and manifest as withdrawal (Fig. 1.2D). The exhibition of withdrawal signs, following the removal of chronic morphine, is categorized as physical dependence [25,28,30,31].

The physiological implications of MOR signaling are widespread, as MOR is distributed throughout both the central nervous system (CNS) and the enteric nervous system (ENS) [32]. The primary analgesic effect of opioids is a result of reduced synaptic transmission in ascending and descending nociceptive circuits. Primarily, reduced transmission from the periaqueductal gray neurons projecting to the rostral ventral medulla and locus coeruleus results in analgesia- the inability to perceive pain [33]. Chronic morphine administration results in analgesic tolerance- the loss of therapeutic effect at the original dose [34]. This results in the need for dose escalation to achieve therapeutic levels of pain relief. Analgesic doses of morphine also reduce the rate of respiration through MOR action in the pre-Bötzinger complex neurons, a small portion of neurons in the pons that controls respiratory rhythm [35]. However, tolerance to the respiratory suppressive effects of morphine is not observed. A lack of tolerance to the respiratory depressive effects but profound tolerance to the analgesic effects of morphine increases the risk of respiratory depression death. This is especially problematic in persons with an OUD- where tolerance to the analgesic effects is profound. Like the effects on respiratory function, tolerance to the constipation effects develops more slowly than to the analgesic effects of morphine [36]. Opioid receptors in the ENS inhibit contraction of the smooth muscle of the myenteric ganglia, leading to constipation. This leads to chronic constipation and in severe cases, narcotic bowel syndrome [32]. It also has a profound effect on the microbiome and disrupts the gut-brain axis as well as inducing dysbiosis [37,38].

MOR receptors in the mesolimbic dopamine system produce feelings of euphoria. This evolutionarily conserved circuit is part of the motivational system that encourages organisms to seek rewards in food, drink, sex, and social interaction. Opioids mimic these naturally derived feelings of pleasure by increasing dopamine in the Nucleus Accumbens (NAc), a component of the mesolimbic dopamine system (Fig. 1.3A) [39]. Opioids accomplish this by inhibiting GABA neurons that synapse onto the

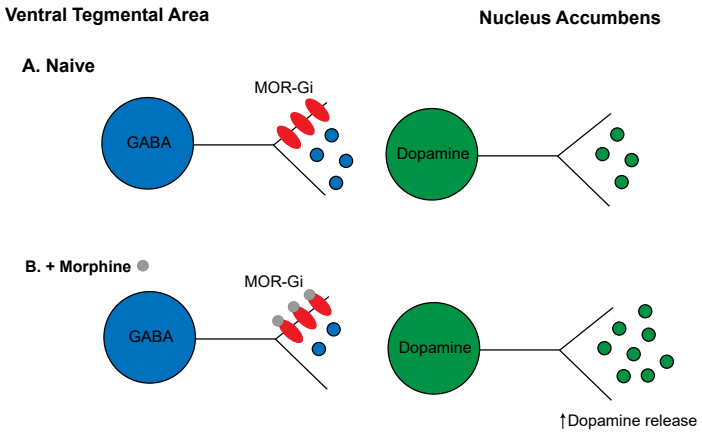

Fig. 1.3- Opioids act as drugs of abuse by increasing dopamine release in the nucleus accumbuns. A) A naive circuit shows a GABA neuron (blue) with opioid receptors (red) on the terminal. GABA neurons release GABA (blue) onto GABA receptors on dopamine neurons (green). Dopamine neurons release dopamine molecules in the nucleus accumbens (green). B) Opioids inhibit the release of GABA, reducing inhibitory GABA input on the dopamine neuron and increasing dopamine.

Ventral Tegmental Area (VTA) (Fig. 1.3A). Disinhibition of the DA neurons projecting from VTA and ending in the NAc leads to an increase of DA in the NAc (Fig. 1.3B). Repeated strong stimulus of these pathways leads to alterations in firing patterns known as long term potentiation (LTP). With continued drug intake, these reward pathways become dysregulated through LTP and changes in neuronal structure and connectivity. These changes have been demonstrated in multiple brain regions [40]. For example, CREB-mediated neuroadaptations in the locus coeruleus most likely underly physical dependence, as CREB knockout mice exhibit decreased states of withdrawal [25]. Changes in the hippocampus, amygdala, and prefrontal cortex lead to drug-stimulus pairing, cognitive dysfunction, and other characteristic brain changes.

Morphine is an incredibly effective analgesic acutely, but chronic use of morphine presents severe limitations. The complexity of this issue is increased by the wide distribution of MOR throughout the CNS and ENS, affecting multiple organ systems. The increasing doses needed to maintain analgesic efficacy increase the risk of respiratory

depression and constipation, as well as the likelihood of inducing irreversible changes in neuronal networks. Physical tolerance results in withdrawal upon cessation of morphine- leading to opioid withdrawal syndrome. Symptoms of opioid withdrawal syndrome are highly unpleasant (see Table 1) and prevent individuals on chronic opioids from discontinuing use. Therefore, prevention of tolerance and dependence is crucial to the development of opioids with reduced side effects. However, the exact relationship between acute signal transduction and chronic adaptations to the consolidation of these changes into downstream neurocircuitry is unclear (Fig. 1.4).

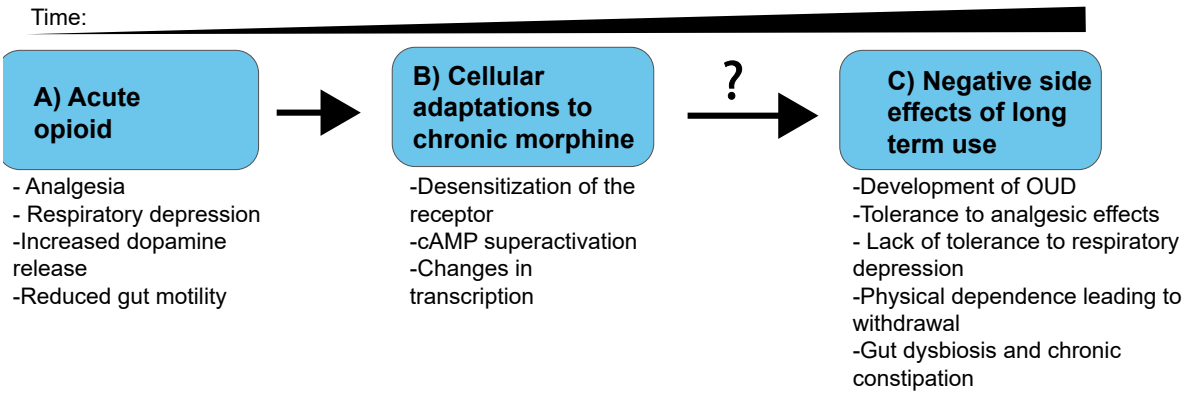

Figure 1.4- The translation of acute and chronic mechanisms of morphine action on the negative side effects of opioids. A) Effects of opioids acutely. B) Cellular adaptations to chronic morphine. C) Several negative side effects of long-term opioid use persist, but there is no clear consensus on the translation of cellular adaptations on these side effects (?)

III) Prevailing theories on the improvement of opioid signaling profiles

Multiple competing theories exist about how to create an opioid with an improved effect/side effect profile. The discrepancies among these theories lie in a central controversy: the role of Arr3 in opioid signal transduction. When MOR is activated by its endogenous ligand, endorphin, it effectively activates G-protein and recruits Arr3. This is defined as a ‘balanced agonist’. However, research indicating that arr3-ko mice had reduced analgesic tolerance [41] and respiratory depression [42] led to the idea that Arr3 may mediate other negative side effects. The field began to steer the development

of ligands away from those that recruited Arr3. Ligands that preferentially signal to one effector over the other are labeled as ‘biased agonists’. Many opioid analgesics, including morphine and oxycodone, efficiently activate G-protein but do not sufficiently recruit Arr3, making them biased. New molecules including oliceridine, PZM21, and SR-17019 were specially designed to be G-protein-biased agonists [43,44]. Oliceridine is the only new pharmaceutical opioid on the market to be clinically tested and was initially reported to induce less respiratory depression and constipation [45]. Despite initial promise oliceridine was not approved by the FDA in 2018 due to its clear abuse liability and failure to show significant improvements over morphine.

One alternative theory states that low intrinsic efficacy opioid ligands offer a beneficial effect/side effect profile. In Gillis et al, they determined the intrinsic efficacy at MOR by measuring direct readouts of $G_{\alpha i}$ signaling, including detection of the active conformation of MOR with NB33 recruitment, direct Gi recruitment, G alpha activation, and cAMP inhibition [46]. They then quantified Arr3 recruitment directly, as well as using GRK2, and measured early endosome formation. They then calculated efficacy values at each effector (G-protein and Arr3) and found that efficacy for G-protein and Arr3 are highly correlated, indicating previously determined bias factors are incorrect. This is supported by the observation that reduced side effects seen in response to oliceridine, PZM21, and SR-17018 which were previously attributed to bias against Arr3 recruitment, are due to low intrinsic efficacy [47]. The authors argue that low intrinsic efficacy explains the beneficial side effect profile of buprenorphine and argues that further development of opioids with low intrinsic efficacy should be considered. These results are supported by the observation that highly potent agonists such as fentanyl and new semi-synthetic analogs such as remifentanil cause profound respiratory depression. Overdose and death have increased exponentially since the advent of these high-affinity drugs, eliminating them as an avenue for future development. One critique of this argument is that it assumes that intrinsic efficacy in one tissue predicts its

function in another tissue. The intrinsic efficacy of signal transduction from MOR may be significantly altered in brain regions and respiratory centers. Lower intrinsic efficacy of the agonist in respiratory centers could result in a lack of neuronal firing decisions in the respiratory systems, whereas high-affinity agonists do cause firing in these regions.

A second prevalent theory states that preventing desensitization of the receptor will prevent the development of cellular tolerance [48]. Morphine results in phosphorylation at residue S375 of the c-terminus barcode, but not residues T370 and T376/9 [13,14]. Further desensitization of the receptor by PKC phosphorylation of S363 occurs as well (Fig. 1.1D). This partial phosphorylation is insufficient to cause subsequent internalization of MOR, leaving receptors present on the membrane G-protein, but does not halt signaling entirely. Evidence for this theory includes that deficiencies can be circumvented by overexpression of GRKs and result in complete phosphorylation[49,50]. An additional layer of complexity ensues when one considers that expression levels of GRKs and Arr3 vary by brain region. This most likely explains the range of phosphorylation states shown in response to morphine in different brain regions. One major critique of this theory is that it fails to explain cAMP superactivation (Fig 1.2D). As receptor desensitization alone is unlikely to affect cAMP levels, it does not explain how withdrawal signs, which are precipitated by a sharp increase in cAMP, would persist.

Another theory- ‘functional selectively’ postulates that opioids need to mimic the endogenous signaling profile, making them balanced ligands [51]. However, robustly defining a drug’s bias profile to develop a balanced ligand has proven difficult. The operational model of agonism is used to quantify bias and requires estimation of the intrinsic efficacy of a ligand for a given pathway. Although quantification of Arr3 recruitment is straightforward, quantification of G-protein assays frequently measures amplified signaling making it difficult to quantify the small upstream change in G-protein signal. The presence of spare receptors also complicates the assessment

of agonist bias for high efficacy ligands that need only to activate a portion of the available receptor pool to achieve the maximal response. In addition to significant challenges in determining bias, separating drug effect/side effects from differences in pharmacokinetics and pharmacodynamics make direct comparisons of drugs impossible. For example, methadone appears to have a bias profile closest to that of endorphin but large amounts of efflux through p-glycoprotein transporters out of the CNS in combination with variable metabolism prevent it from having more clinical utility [52].

To circumvent the inherent complications in interpretation due to pharmacokinetic/pharmacodynamic differences, Whistler et al 2008 utilize a mouse model with mutation of the MOR receptor itself [53]. The RMOR mouse (recycling Mu-Opioid Receptor) has 28 amino acids of the C-tail from the delta opioid receptor, increasing the affinity for GRKs [27]. Subsequently, robust endocytosis of the RMOR in response to morphine is observed. This model effectively increases Arr3 recruitment (See Table 2). The RMOR mice show reduced development of tolerance to chronic morphine treatment, as well as reduced signs of physical dependence [53]. Interestingly, RMOR mice show increased reward in response to morphine, but reduced abuse liability [54]. In addition, RMOR mice did not show enhanced respiratory depression in response to WT mice [55]. These data indicate that increased Arr3 recruitment improves tolerance and dependence without decreasing analgesic efficacy.

Further evidence from phospho-null mice (10S/T-A, 11S/T-A) contribute to the complexity of these competing theories [50]. Phospho-null mice have potential GRK phosphorylation sites by mutating residues to alanine, preventing any regulatory phosphorylation events, and similarly decreasing Arr3 recruitment. This model shows a 1.5x reduction in the level of tolerance to chronic morphine, a but similar level of physical dependence. These data indicate that reduced Arr3 recruitment improves the development of tolerance. This is in conjunction with previous findings from arr3-

KO mice exhibiting reduced tolerance and respiratory depression, but similar level of physical dependence. This is contradictory if one considers that both the removal of Arr3 (arr3-KO, phospho-null) and the enhanced recruitment of Arr3 (RMOR) both seem to reduce analgesic tolerance. In addition, both RMOR and arr3-KO have increased reward sensitivity and analgesic potency and conflicting results on respiratory depression.

Genotype	Acute analgesia	Reward	Analgesic Tolerance	Physical Dependence	Signaling Summary:	Signaling bias:
WT	+	+	+	+	Arr-3, GRKs, and phosphorylation sites present but arr-3 recruitment is minimal	biased for G protein
Recycling MOR (RMOR)	++	++	- -	-	Arr-3, GRKs, and phosphorylation sites present. Arr-3 recruitment is robust.	balanced
Arrestin3-KO	++	++	-	+	GRKs and phosphorylation sites present. Arr-3 recruitment is not possible	biased for G protein
MOR phospho-null (11S/T-A)	+	+	-	+	Arr-3 and GRKs present. Arr-3 recruitment is not possible due to lack of phosphorylation sites, but Arr-3 is present	biased for G protein

Table 2- Summary of opioid effects and side effects in mouse models of biased agonism at MOR. WT responses for acute analgesia (Effect), reward, analgesic tolerance, and physical dependence (side effects), top row. WT responses are defined as a single plus while increased response is denoted as multiple plus signs. Decreased responses are denoted as minus signs, while multiple minus signs indicate even lower responses. Responses for RMOR, Arr3-KO, and MOR phosphor-null mice are listed in subsequent rows. A summary of the signaling profile modeled by each genotype and its characterization as biased or balanced is listed on the right.

IV) Methodology and project summary

Opioid pharmaceuticals remain the most effective drugs for severe pain, but negative side effects such as the development of OUD limit their therapeutic utility. Improvement of opioid pharmaceuticals to limit the development of these negative side effects could be accomplished in several ways: 1) Improvement of the cellular signaling profile of opioids to reduce the risk of OUD, 2) Mitigation of the percentage of persons who transition into OUD. Firstly, I propose the improvement of the cellular signaling profile induced by opioid pharmaceuticals by answering a long-standing question: The role of Arr3 in the development of drug-seeking behavior. Specifically, is the recruitment

of Arr3 and subsequent endocytosis of MOR beneficial to regulating signal transduction, or does Arr3 mediate drug-seeking behavior? Secondly, I use the data generated above to predict if anxiety state or microbiome composition are predictive of which individuals transition into OUD.

To answer these questions, I developed a rodent model of addiction behavior that occurs in three phases: 1) transition to compulsive drug use, 2) difficulty in ceasing drug-seeking behavior, and 3) relapsing drug use after abstinence. The weekly operant self-administration models escalation in drug use seen in human pathology, where drug-seeking behavior transitions from impulsive to compulsive after repeated exposure. The extinction phase models difficulty in ceasing drug-seeking behavior, while reinstatement models relapse of drug use after a period of drug use. Self-administration allows for personality differences to dictate the amount being administered, whereas previous studies with forced administration collapse mice into equal exposure. This allows us to differentiate mice into categories of those who are high drug seekers (compulsive) and those who are not (non-compulsive). To accomplish this, a composite compulsivity score is calculated for each animal based on its behavior in each of the three phases. I utilized three genotypes of mice in this self-administration paradigm: RMOR (increased ability to recruit Arr), arr3-KO (no ability to recruit Arr), and WT mice. These data will determine the role of Arr3 in drug-seeking behavior. If Arr3-KO mice present a similar degree of drug-seeking behavior as WT mice, then Arr3 does not mediate drug-seeking behavior. Similarly, if RMOR mice show reduced drug-seeking behavior compared to WT mice, Arr3 would be beneficial in the prevention of OUD.

I then sought to determine if anxiety state, social hierarchy, or changes in microbiome composition are predictive of which individuals transition to OUD. Human development of OUD is a complex interaction between genetic predisposition, environmental factors, and neurobiological changes that make resisting drugs in

the future more difficult. In a homologous population of mice, genetic and certain environmental factors are held constant, but a subset of mice still develop compulsive tendencies. With genetic variability and environment controlled for in a laboratory setting, these differences must be the result of individual predispositions in mood/personality or differential adaptations in response to drugs. I assessed differences in anxiety states by conducting a battery of five anxiety measures both before admission onto the paradigm (baseline anxiety) and following the last exposure to morphine (morphine withdrawal). I also assessed differences in personality by conducting a social hierarchy assessment to determine if inherent social order predisposes mice to become compulsive drug users. Finally, in collaboration with Dr. C. Whistler's lab at the University of New Hampshire, we assessed changes in microbiome composition in response to morphine to determine if gut dysbiosis modulates drug-seeking behavior.

I hypothesize that deletion of Arr3 will not decrease drug-seeking behavior, indicating that Arr3 does not mediate the negative side effects of opioids. I predict that increased anxiety and dominant social hierarchy will predict OUD development and that profound changes in microbial composition will be observed in mice that are compulsive drug seekers. These findings have implications for the development of new opioids, as well as the treatment of OUDs: 1) Inclusion of the development of balanced agonists that recruit Arr3, 2) determination of key microbial changes can be counteracted with probiotic supplementation, and 3) determination of the direction of causality between anxiety and OUD development to inform treatment and identify risk factors in human populations.

Chapter 2

Deletion of arrestin-3 does not alter compulsive morphine-seeking behavior in an oral operant self-administration paradigm

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In submission.

ABSTRACT

Opioid drugs are potent analgesics that mimic the endogenous opioid peptides, endorphins and enkephalins, by activating the μ-opioid receptor. Opioid use is limited by side effects, including significant risk of opioid use disorder. Improvement of the effect/side effect profile of opioid medications is a key pursuit of opioid research, yet there is no consensus on how to achieve this goal. One hypothesis is that the degree of arrestin-3 recruitment to the μ-opioid receptor impacts therapeutic utility. However, it is not clear whether increased or decreased engagement of the μ-opioid receptor with arrestin-3 would reduce compulsive drug seeking. To examine this question, we utilized three genotypes of mice with varying abilities to recruit arrestin-3 in response to morphine in a novel longitudinal operant self-administration model. We demonstrate that drug-seeking behavior in arrestin-3 knockout and wild type mice are indistinguishable. In contrast, in mice where the μ-opioid receptor strongly recruits arrestin-3, drug-seeking behavior is reduced. Our data suggest that opioids that engage both G protein and arrestin-3, recapitulating the endogenous signaling pattern, will reduce abuse liability.

INTRODUCTION

Opioids are highly effective analgesic drugs that remain essential for the treatment of severe pain. Despite their therapeutic utility, opioid use can precipitate opioid use disorder (OUD), with 2.7 million adults in the US living with OUD[51]. Despite significant efforts and billions of dollars, the development of an opioid with reduced abuse liability has been ultimately unsuccessful. This lack of success stems in part from a poor understanding of the signaling attributes responsible for mediating the neuroplasticity that underlies OUDs.

All opioids exert their analgesic actions primarily by activating the μ-opioid receptor (MOR), a G protein-coupled receptor (GPCR) [52]. Endogenous opioid peptides, endorphins and enkephalins, bind and activate MOR to promote signaling

to the $G_{i/o/z}$ G protein effectors. G protein signaling from these peptide-occupied MORs is then titrated by a cascade of events that includes phosphorylation of the MOR by GPCR kinases (GRKs) at four distinct residues [12,17] and recruitment of the arrestin-3 (β-arrestin-2) effector to the phosphorylated receptor [53]. Arrestin-3 recruitment not only uncouples MOR from G protein, thereby “arresting” G protein signaling but also promotes MOR endocytosis [54,55]. Endocytosed MORs are then dephosphorylated and recycled to the plasma membrane where they can bind ligand and initiate another cycle of signal transduction [56,57]. In contrast, while activation of the MOR by opioid drugs, including morphine and all its derivatives, also promotes G protein signaling, the morphine-activated receptors only weakly engage the GRK and arrestin-3 effectors [12,19,58], with phosphorylation of the MOR limited to one of the four residues [17] unless GRKs [53] and/or arrestins [59] are highly expressed. The endogenous opioid peptides are thus “balanced” ligands that engage both the G protein and arrestin effectors while the opioid drugs are often “biased” ligands that favor G protein signaling in many cell types.

The impact of biased versus balanced signaling on the effect/side effect profile of opioid analgesics has been interrogated for more than two decades since the original discovery that morphine does not promote MOR endocytosis [18,60]. Nevertheless, there remains little consensus on whether arrestin-3 recruitment increases or decreases the negative side effects of opioids. This controversy persists because both eliminating arrestin-3 recruitment and enhancing arrestin-3 recruitment reduce some of the side effects of morphine. For example, mice with a disruption of the arrestin-3 gene (Arr3-KO) were reported to show increased analgesia [35], reduced tolerance [61], and reduced respiratory depression and constipation [36] in response to morphine compared to wild type (WT) mice. Likewise, knock-in mice where the MOR is replaced by a mutant receptor where all 11 potential phosphorylation sites are mutated to alanine (MOR 11S/T-A) are also reported to show improved analgesia and reduced analgesic

tolerance, but no difference in respiratory depression in response to morphine [44]. These data would suggest that removing arrestin-3 engagement improves analgesic utility. However, promoting the recruitment of arrestin-3 by replacing the MOR with a mutant receptor that is a better substrate for GRKs (RMOR mice, for recycling MOR), was also reported to produce enhanced analgesia [62] and reduced analgesic tolerance to morphine with no change in respiratory depression [50]. Thus, both increasing and decreasing arrestin-3 recruitment in response to morphine improves analgesia outcomes. In addition, both decreasing (Arr3-KO mice) [63] and increasing (RMOR knock-in mice) [49] arrestin-3 recruitment were reported to show increases in the potency of morphine reward in conditioned place preference (CPP) paradigms. Dependence, defined as physical and/or affective signs of distress upon the removal of drug, is another negative side effect of opioid use and key component of OUDs. Both mouse lines deficient in arrestin-3 recruitment (Arr3-KO, MOR 11S/T-A) were reported to show intact or exacerbated morphine withdrawal signs, indicating that they still develop dependence [44,61]. In contrast, RMOR mice show neither physical [62] nor affective [49] signs of dependence upon withdrawal from morphine. This battery of conflicting results has left the field with no consensus on the best therapeutic strategy for new opioid drugs.

Perhaps the most treatment-limiting liability of opioid analgesics is the development of addiction, a syndrome in humans defined by a constellation of phenotypes that include loss of control in drug-seeking behavior, craving, and relapse. We have previously reported a three-phase operant self-administration paradigm that models compulsive drug seeking in mice: escalation of drug seeking (loss of control), failure to extinguish drug seeking (craving), and reinstatement after prolonged abstinence (relapse). Using this model, we demonstrated that WT but not RMOR mice become compulsive drug seekers with time [49]. However, no one has yet assessed how eliminating arrestin-3 engagement with MOR impacts compulsive drug seeking.

Since many of the side effects of opioids are improved with both the enhancement and the elimination of arrestin-3 to the MOR, the predicted answer to this question is unclear. Whether MOR engagement with arrestin-3 prevents or exacerbates compulsive drug seeking risk is a critical question, as the answer could inform the best strategy to develop new opioid medications with reduced abuse liability. To answer this question, we utilized our compulsive drug seeking model in mice of three genotypes with variable arrestin-3 engagement in response to morphine: WT, Arr3-KO, and RMOR.

METHODS

Mice

Mice of 3 genotypes were used in this study: 1) C57Bl/6 WT (n=26, 19 male, 7 female, 8 bred in-house and 18 purchased from the Jackson Laboratory) 2) RMOR [62] (n=15, 8 male, 7 female) bred in house, congenic >30 generations on C57Bl/6 and 3) Arr3-KO (n=16, 7 male, 9 female) originally acquired from Dr. R. Lefkowitz (Duke University) [35] and bred in-house congenic for >30 generations on C57Bl/6. Adult mice aged 9-11 weeks at the start of training were used. Mice were singly housed with running wheels as extra enrichment upon entering the study and had access to food and water *ad libidum*. Single housing was necessary to monitor morphine consumption in the home cage. Mice were housed in a room with a reversed 12-hour dark/light cycle so that all study tasks took place during their active/dark period.

Operant Training with saccharin reward

Med Associates operant conditioning chambers (Fairfax, VT) were used for the extent of this study. Mice were first trained to press a lever for a reward using saccharin as the reinforcer. Both active and inactive levers were present at the start of training. The active lever was indicated by the presence of a light cue above the lever while inactive levers were unlit. A press on the light-cued active lever delivered 15 µl of 0.2% saccharin sodium salt hydrate (Sigma-Aldrich, St. Louis MO) that was signaled by the illumination

of a cue light above the delivery port and a 2.5-second tone (see Fig. 2.1C). Mice were trained in two stages: Stage 1 consisted of a progressive fixed ratio (FR) reinforcement schedule from FR1 (every active lever press produces a reward) to FR4 (four consecutive presses are required to produce a reward). Mice had to obtain 20 rewards at each FR step before progressing to the next step. Hence, to pass Stage 1 mice had to press a total of 200 times for 80 rewards (20 at FR1, 40 at FR2, 60 at FR3, and 80 at FR4). Each session lasted a maximum of 6 hours. Mice that failed to pass Stage 1 after 6 sessions were eliminated from the study. In Stage 2, mice were returned to the box for an FR1-FR4 progressive session with one reward at each FR step before progressing to the next step, which was admittance into the study. Hence to pass Stage 2, mice had to press the active lever 10 times for 4 rewards (1 press at FR1, 2 at FR2, 3 at FR3, and 4 at FR4). Only mice that passed Stage 2 within one hour were entered into the study.

Oral Morphine Consumption Schedule

Mice who successfully completed operant training with saccharin were singly housed and their cages were outfitted with two bottles, one with water and the other with morphine sulfate (MS) (Mallinckrodt Pharmaceuticals, St. Louis, MO) + 0.2% saccharin to counteract the bitter taste of MS. In addition, to acclimate mice to the bitter taste of MS, in week 1 the concentration of MS was 0.3 mg/mL, in week 2 MS was 0.5 mg/mL, and in weeks 3-17 MS was 0.75 mg/mL. Mice had access to both the MS bottle and the water bottle 5 days per week and water only for the two days preceding their operant session. MS and water bottles were weighed three times a week to monitor total morphine consumption. The vast majority of morphine consumption that occurred was in the home cage, as each mouse was free to self-administer as much morphine as they choose 5 days per week.

Operant Oral Self-Administration Weekly Schedule

During weeks 1-17, mice remained on the same weekly schedule (Fig 2.1B). After two days of access to only water, mice were placed in the operant box for a 30-minute

session (Peach bars, Fig 2.1B) that consisted of two distinct phases. Phase 1 was a time-out period (TO) and Phase 2 was a variable interval period (VI). The TO period was signaled by the presence of a flashing light above the active lever and no light above the inactive lever. No lever presses were rewarded during this 5-minute TO period, which in our OUD model reflects “futile drug-seeking”. After the 5-minute TO, the light above the active lever stopped blinking and remained on, initiating the start of the 25-minute variable interval reward period. During this VI period, the first active lever press was rewarded by delivery of a 15 µl morphine reward (0.5mg/mL MS in 0.2% saccharin), paired with the illumination of the light above the port and a 2.5-second tone. After that first reward, the wait time necessary between available rewards was unpredictable, from 1 to 90 seconds, but averaged 25 seconds. Time intervals for the VI were randomly selected from a 12-element Fleshler–Hoffman series to ensure all mice could access the same number of rewards. In a VI schedule, the total amount of drug available to be earned is thus held constant, and there is no precise relationship between the number of responses emitted by the mouse and the number of reinforcers received. In our OUD model, the VI schedule captures rates of lever pressing that reflect how hard a mouse is willing to work for drug since not all presses produce reward. All lever presses and all rewards consumed were automatically recorded during this weekly 30-minute operant session (Fig. 2.1C). After the operant session, mice were returned to their home cage with free access to both water and the morphine bottle for the next 5 days. This was followed by two days of water only and the next operant session for a total of 17 weeks. This stage of our paradigm models the transition from reward-based lever pressing to compulsive drug-seeking.

Extinction

Extinction began in week 18. Three 30-minute extinction sessions were conducted every day for a maximum of 12 days (Green bars, Fig 2.1B). Extinction sessions were identical to operant sessions except for lever presses on the active lever during the VI

period did not deliver a morphine reward or the associated tone and light cues. Mice were considered extinguished when their 3-session average of active lever presses for the day was less than 20% of their active lever presses during weeks 15-17 of their operant responding or after 12 days of extinction, whichever came first. Hence, some mice had more extinction sessions than others. All lever presses during these extinction sessions as well as days to extinguish were automatically recorded (Fig. 2.1C). During the extinction training, mice had access to only water (no morphine) in their home cage. This stage of our paradigm is designed to model difficulty in stopping drug-seeking.

Reinstatement

Following extinction, mice were returned to their home cage with access to only water for two additional weeks with no morphine access (light purple bar, Fig. 2.1B). Following this abstinence period mice were returned to the operant box for a single operant session. This session consisted of a 5-minute TO period identical to previous sessions. After this TO period, the light over the active lever remained on and a single non-contingent (no lever press required) morphine reward was delivered to the port together with the light and sound cues. After this single reward delivery, the light remained on over the active lever and levers could be pressed, but no additional rewards were delivered. This stage of our paradigm is designed to model both cue- and drug-induced relapse. During this session, all lever presses, all head port entries, and the latency to collect the non-contingent reward were recorded (Fig. 2.1C).

Scoring OUD/compulsivity

A total of 16 measures from throughout the paradigm were used to create a composite OUD/compulsivity score for each mouse (see Fig. 2.1C for the variables used in the final score). To create this composite score for variables with significantly different raw values (lever presses on the active lever outnumber those on the inactive, for example), the raw values for each mouse for each of these 16 measures were Z-scored across the population of mice that completed the study (58 mice: 26 WT, 15 RMOR, 16 Arr3-

KO). A sub-score for each of the three phases (operant, extinction, reinstatement) was then created by averaging the Z scores of each behavior in that phase for each mouse thereby giving each mouse an operant, extinction, and reinstatement sub-score where each behavior in that phase is equally weighted. A final “compulsivity score” for the entire paradigm was then created by adding the operant, extinction, and reinstatement sub-score for each mouse. Categorical assignments of compulsive or not compulsive were determined using the mean and interquartile standard deviation (IQD) composite score of WT mice. The IQD is defined as the standard deviation between Q1 and Q3. Mice with a composite score of 1 IQD or more over the mean of WT mice were designated as compulsive.

Preference

On days 3-5 of week 17, we conducted a preference test for morphine (sweetened with 0.2% saccharin) versus saccharin alone. To do this, the water bottle in the home cage was replaced with a bottle of 0.2% saccharin for 4 hours during the dark cycle and consumption of both morphine and saccharin was determined by weighing the bottles before and after this test. Preference for morphine over saccharin was calculated with the formula: MS consumed (in mLs)/Total fluid consumed (in mLs).

Study Approval

All protocols were approved by the Institutional Animal Care and Use Committee at the University of California Davis and are in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

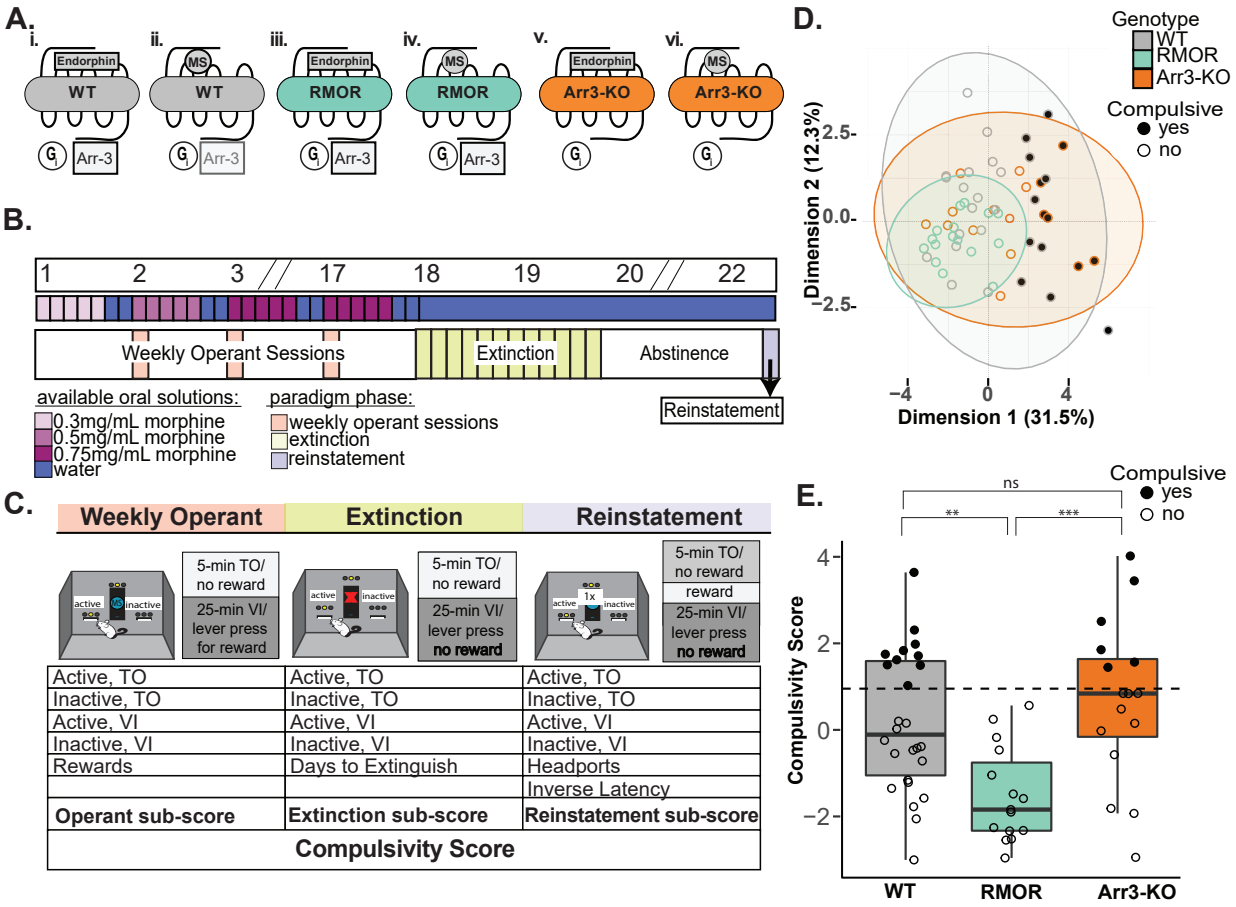

Figure 2.1- Modeling compulsive morphine seeking in WT, RMOR, and Arr3-KO mice. A) schematic of MOR signaling in WT, RMOR, and Arr3-KO mice in response to morphine and the endogenous ligand, endorphin. Effectors include $G_{i/o/z}$ protein (G_i, circle), Arrestin-3 (Arr3, square) B) Experimental paradigm. Top bar shows weeks where slashes indicate large gaps in the timeline. Colored blocks represent oral morphine sulfate (MS) availability in the home cage where water only is blue and increasing concentrations of MS (0.3 mg/mL, 0.5 mg/mL, and 0.75 mg/mL MS) are lightest to darkest pink (middle bar). Mice had access to morphine and water five days a week and water only the two days preceding the operant lever pressing task (see methods). Bottom shows the three phases of the paradigm. Phase 1: 17 weeks of home cage drinking, with an operant session one day per week (peach bars). Phase 2: Lever pressing behavior was extinguished in up to 12 extinction sessions (green bars). Phase 3: Cue-induced reinstatement (light blue bar) of lever pressing following a 14-day drug-free period. C) Variables included in compulsivity scores for each phase of the paradigm. Top panel shows a schematic of operant box conditions during each phase. Bottom table shows behaviors from each paradigm phase that were included in the composite compulsivity score. Lever press types include active and inactive presses in both the initial 5-minute timeout (TO) period followed by the 25-minute variable interval (VI) period. D) Principal component analysis of behaviors throughout the paradigm show that RMOR (teal) mice cluster tightly while WT (gray) and Arr3-KO (orange) mice

have more variable behavior. All the compulsive mice (filled grey and orange circles) fall outside the RMOR cluster. E) Final composite compulsivity scores for each mouse. Scores of compulsive mice (closed circles) were one interquartile deviation above the mean compulsivity score of WT mice. Non-compulsive mice (open circles) were defined as all mice below this threshold. All RMOR mice fall below the threshold. WT mice significantly differed from RMOR mice, but not from Arr3-KO mice in a one-way ANOVA with Tukey's multiple comparisons correction (p=0.0052, p=0.57 respectively). Arr3-KO mice were significantly different than RMOR mice (p=0.0009).

RESULTS

Deletion of Arr3 does not alter compulsive drug-seeking.

To determine whether the degree of arrestin-3 (Arr3) recruitment to MOR modulates compulsive drug-seeking, we compared WT, RMOR, and Arr3-KO mice in a longitudinal mouse model of OUD. These three genotypes are differentiated by their arrestin-3 recruitment to MOR. In WT mice, MORs recruit arrestin-3 in response to endorphin/enkephalin activation but only weakly in response to morphine activation (Fig 2.1Ai,ii). In RMOR mice, the receptor recruits arrestin-3 in response to both endorphin and morphine activation [48] (Fig 2.1Aiii,2.1Aiv). In Arr3-KO mice, the MORs have no ability to recruit arrestin-3 (schematic in Fig 2.1Av,1Avi).

Briefly, to monitor the transition to compulsive drug-seeking and relapse as described previously [49], we combined elements of operant self-administration and a two-bottle choice test. This longitudinal paradigm consisted of three separate stages: 1) Weekly Operant 2) Extinction and 3) Reinstatement (Fig 2.1B). We quantified 16 behavioral measures across the paradigm (Fig. 2.1C). A Principal Component Analysis (PCA) of these 16 measures revealed that RMOR mice cluster tightly together while both WT and Arr3-KO behavior have high variability (Fig 2.1D). We hypothesized that the variability in the WT and Arr3-KO populations reflected that some mice of those genotypes had become "compulsive". To examine this hypothesis, we calculated a composite compulsivity score for each mouse that incorporated all 16 measures (see methods for details) and reflected their behavior relative to the entire population (Fig. 2.1E). We then determined which mice were outliers and designated these mice as

compulsive. All compulsive mice fell outside of the RMOR cluster in the PCA (Fig. 2.1D solid circles). Of the 26 WT mice, 10 (38.5%) were compulsive. Of the 16 Arr3-KO mice, 6 (37.5%) were compulsive. None of the 15 RMOR mice were compulsive, replicating what we have previously shown [49]. Comparison of composite compulsivity scores showed a significant genotype effect (Fig. 2.1E, p=0.0008, F=8.220, one way ANOVA). When comparing composite compulsive scores between each genotype, WT and Arr3-KO mice show no significant difference in compulsivity (p=0.57) but both WT and Arr3-KO show a significant difference in compulsivity from RMOR mice (p=0.005, p=0.0009, respectively, adjusted p-values from a one-way ANOVA with Tukey's multiple comparisons) (Fig. 2.1E). These data indicate that preventing arrestin-3 engagement does not reduce compulsive drug-seeking and that effective arrestin-3 engagement diminishes the liability for compulsive drug-seeking.

Compulsive mice show altered lever pressing behavior in late operant, extinction, and reinstatement phases but not in their early operant phase.

We next assessed at what point in the paradigm the compulsive and non-compulsive mice diverged in behavior. Behavior during each paradigm-specific phase was assessed using a two-way ANOVA with Tukey's multiple comparisons and reported p-values are adjusted (Fig. 2.2A-D). During the first three weeks of operant responding, there was no significant difference in the lever-pressing behavior between WT mice that were later identified as compulsive and WT mice that were later identified as non-compulsive mice (Fig. 2.2A, left gray bar vs. right gray bar). Similarly, there was no significant difference between non-compulsive Arr3-KO mice and compulsive Arr3-KO mice (Fig 2.2A, left orange bar vs. right orange bar). Although these values were trending towards significance (p=0.06), they did not differentiate compulsive and non-compulsive mice and were therefore not included in composite compulsivity scores.

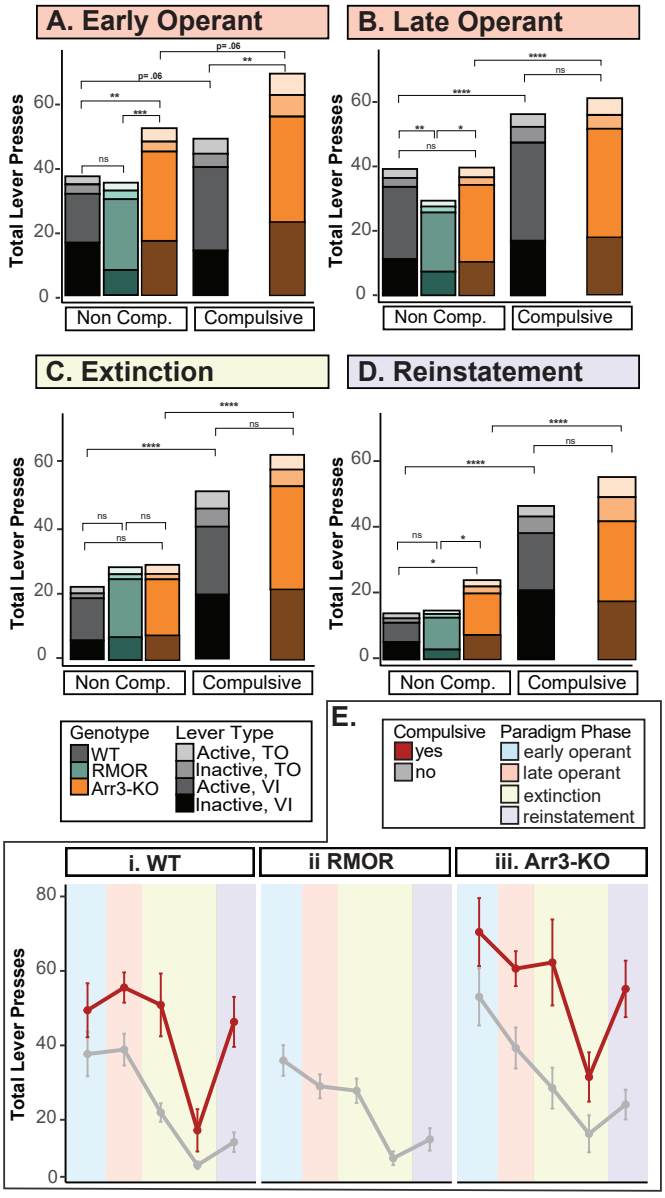

Figure 2.2- Changes in morphine seeking through time. A) Lever pressing during the early operant phase (weeks 1-3). Compulsive and non-compulsive mice do not differ in their lever pressing behavior early in the paradigm. Bar height displays total presses during the 30-minute operant session. The color gradient indicates each lever press type (lightest to darkest: active and inactive lever presses during the timeout (TO), active and inactive during the variable interval (VI). B) Lever pressing during the late operant phase (weeks 15-17). Compulsive mice show increased lever pressing compared to non-compulsive mice (****p ≤ 0.0001). Bar height and color gradient as in A for panels B-D. C) Extinction. Compulsive mice show increased lever pressing during extinction compared to non-compulsive mice (****p ≤ 0.0001). D) Reinstatement. Compulsive mice show an increase in lever pressing in reinstatement compared to non-compulsive mice (****p ≤ 0.0001) (ns: p > .05, * p ≤ 0.05, ** p ≤ 0.01, *** p≤ 0.001, ****p ≤ 0.0001). All comparisons were made using a two-way ANOVA with Tukey's multiple comparisons test; p-values shown are adjusted. E) Summary longitudinal comparison of the data depicted in A-D for compulsive (red) vs. non-compulsive (gray) mice for each genotype in early operant, (light blue), late operant (light red), extinction day 1 and last day (light green), and reinstatement (light purple).

However, by the late operant phase (weeks 15-17) there was a significant difference in lever pressing behavior between compulsive and non-compulsive WT mice (Fig. 2.2B, ****p ≤ 0.0001), as well as between compulsive and non-compulsive Arr3-KO mice (Fig. 2.2B, ****p ≤ 0.0001). Compulsive Arr3-KO and WT were indistinguishable during the late operant stage (Fig 2B). During extinction, there was also a significant difference between non-compulsive and compulsive WT mice (Fig. 2.2C, ****p ≤ 0.0001), and between non-compulsive and compulsive Arr3-KO mice (Fig. 2.2C, ****p ≤ 0.0001). There was no significant difference between non-compulsive WT, Arr3-KO, and the uniformly non-compulsive RMOR mice (Fig. 2.2C, left gray vs left teal vs left orange bars, ****p ≤ 0.0001). During the reinstatement stage, there was also a difference in lever pressing behavior between non-compulsive and compulsive WT mice (Fig. 2.2D, ****p ≤ 0.0001) and between compulsive and non-compulsive Arr3-KO mice (Fig. 2.2D, ****p ≤ 0.0001), but no difference between compulsive WT versus compulsive Arr3-KO mice (2.2D). Non-compulsive Arr3-KO mice showed more lever pressing than both non-compulsive WT mice and RMOR mice. Overall, the lever-pressing behavior of compulsive mice and non-compulsive mice are distinguishable in every phase of the paradigm, except early operant which only trended towards significance. In addition, compulsive WT and Arr3-KO are indistinguishable from one another in lever pressing behavior in the late operant, extinction, and reinstatement phases of the paradigm. Data from Fig. 2.2A-D is summarized in Fig. 2.2E comparing compulsive mice (red lines) to non-compulsive mice (gray lines) (Fig 2.2E). These data demonstrate that compulsive and non-compulsive mice diverged in their lever pressing propensity after weeks of morphine consumption and that early measures do not reliably differentiate compulsive and non-compulsive mice.

Morphine consumption does not predict compulsive drug-seeking behavior.

The vast majority of the morphine consumption in our paradigm occurred during home cage drinking. Individual mice were highly variable in their weekly morphine

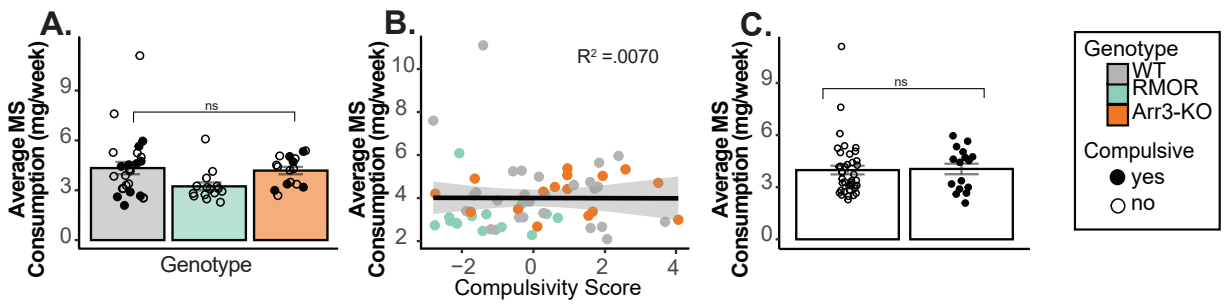

Figure 2.3- Morphine consumption does not predict compulsivity. A) Average weekly consumption in weeks 3-17 in WT (gray), RMOR (teal), and Arr3-KO (orange) mice. There is no significant difference between genotypes (p=0.09, F=2.41, one-way ANOVA) B) Average morphine consumption does not correlate with compulsivity score in a simple linear regression model (p= 0.55, R^2=0.007). C) Average morphine consumption does not differ between compulsive (closed circles) and non-compulsive (open circles) mice (p= 0.73, t=0.34, two-tailed unpaired t-test).

consumption with a range of 2.09 to 11.1mgs consumed per week, on average morphine. There was no significant difference in average morphine consumption (p= 0.09, F=2.41, one way ANOVA) between WT, RMOR, and Arr3-KO mice (Fig. 2.3A). The large range in total morphine consumption per mouse allowed for a linear regression analysis of compulsivity score versus consumption (Fig. 2.3B), which showed no correlation (p= 0.55, R^2= 0.0075). There was also no significant difference in morphine consumption between compulsive and non-compulsive mice, (Fig. 2.3C, p=0.74, t=0.34, unpaired t-test). These data taken together indicate that consumption of morphine does not predict liability for compulsive drug-seeking behavior.

Morphine preference does not predict compulsive drug-seeking behavior.

Another aspect of OUD is the preference of opioid drugs over other sources of positive affect. We assessed whether drug seeking behavior in this paradigm was related to voluntary consumption of morphine (a drug reward) over saccharine (a naturalistic reward). We measured morphine and saccharine consumption over four hours during week 17 of our operant paradigm with home cage morphine access. There was no significant difference in preference for morphine versus saccharin in WT, RMOR and Arr3-KO mice (p=0.36, F=1.03, one way ANOVA) (Fig. 2.4A). Furthermore, preference

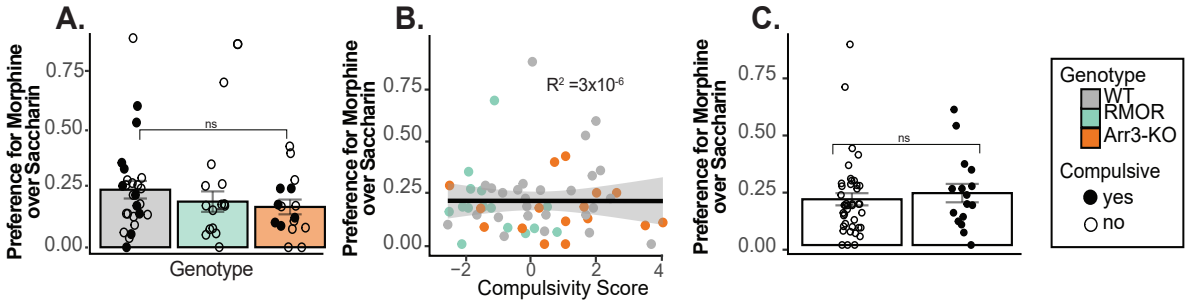

Figure 2.4- Morphine preference does not predict compulsivity. A) Preference for morphine over saccharin for WT (gray), RMOR (teal), and Arr3-KO (orange) mice. Preference for morphine in 0.2% saccharin vs 0.2% saccharin alone was measured on week 17 in a 4-hour two-bottle choice test in the home cage (volume MS consumed/ total volume consumed). Preference did not vary significantly between genotypes (p=0.36, F=1.03, one-way ANOVA) B) Preference for morphine does not correlate with compulsivity score in a simple linear regression model (p=0.98, R^2= 3.637e-006) C) Preference for morphine does not differ between compulsive (filled in circles) and non-compulsive mice (open circles) (p=0.74, t=0.54, two-tailed unpaired t-test).

for morphine did not correlate with compulsivity score (Fig. 2.4B, p=0.98, R^2= 3.637e-006). Additionally, there was no significant difference in morphine preference between compulsive and non-compulsive mice (Fig. 2.4C, p=0.58, t=0.54, unpaired t-test). These data indicate that morphine preference does not predict liability for compulsive drug-seeking behavior.

DISCUSSION

Here we show that deletion of arrestin-3 does not protect against compulsive morphine-seeking in a mouse model of OUD. Specifically, we found that ~38% of both WT and Arr3-KO mice become compulsive drug-seekers. In contrast, none of the RMOR knock-in mice, who express a MOR that strongly engages arrestin-3, transition to compulsive drug-seeking (Fig. 2.1D). Importantly, we also show that neither morphine consumption (Fig. 2.3) nor morphine preference (Fig. 2.4) is predictive of compulsive drug-seeking. Degree of lever pressing early in the paradigm was likewise not predictive of compulsive drug-seeking. However, lever pressing later in the paradigm, difficulty

extinguishing lever pressing, and drug/cue-induced reinstatement were significantly higher in compulsive versus non-compulsive mice (Fig. 2.2). These data indicate that many of the behavioral assays, including simple operant responding, CPP and consumption, used to infer “addiction” in mouse models early in their drug exposure may not be predictive. This aligns with the observation that although reward measured by CPP is enhanced in both RMOR [49] and Arr3-KO [64] mice compared to WT mice, RMOR mice do not transition to compulsive drug-seeking [49] as we reproduce here, while both Arr3-KO and WT mice do. As morphine is rewarding in WT and Arr3-KO mice [63] that develop OUD and in RMOR mice that do not, these data indicate that any future opioids should be evaluated for abuse liability and perhaps not discarded simply because they produce “reward”. Taken together, these data also indicate that G protein-biased opioid ligands, that do not engage arrestin-3 will show no improvement in abuse liability. This is a critical finding as the primary focus of drug development for the past two decades has been towards ultra-G-biased ligands. TRV-130 (Oliceridine) is such an ultra-G-biased ligand and was FDA-approved in 2020—the first new opioid in 4 decades.

In addition to abuse liability, the development of tolerance and dependence present significant limitations to the clinical utility of morphine. The RMOR mice can provide valuable insight into how we might address these undesirable effects as well. In RMOR mice, MOR signaling in response to morphine mimics that of the endogenous opioids by promoting arrestin-3 engagement followed by endocytosis and recycling—in effect converting morphine into endorphin for signaling. RMOR mice show excellent analgesic response to morphine but do not develop tolerance under conditions where both WT [50,62] and Arr3-KO [50] mice do. In addition, RMOR mice do not show either physical [62] or affective [49] dependence while both Arr3-KO [61] and MOR 11S/T-A [44] mice show dependence at a similar level or exacerbated compared to WT mice. Importantly, the pharmacokinetics of morphine—as well as any off-target effects—are unaltered in RMOR knock-in mice and RMOR and WT mice have the same number

of opioid receptors [62]. In addition, the efficacy of morphine and [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO, a hydrolysis-resistant form of enkephalin) are also equivalent both for inhibition of inwardly rectifying potassium channels and for inhibition of transmitter release in the brain [65]. The differentiating characteristic of RMOR mice is that signaling bias in response to morphine has been substantially altered to be more “balanced”, as the MOR is endocytosed and recycled in response to an analgesic dose of morphine [65].

This balance is achieved because the RMOR is a better substrate for GRKs [48], resulting in increased arrestin-3 recruitment in response to morphine. The RMOR receptor is a better substrate for GRKs because it is a chimeric receptor in which 22 amino acids of the cytoplasmic tail of MOR are replaced by those from the closely related delta opioid receptor [48,62]. While creation of this receptor was done stochastically, we now know this sequence replaced the phosphorylation bar code of the MOR [12,13] with that of the DOR making RMOR a better substrate for GRKs so it is more highly phosphorylated when bound to morphine, thereby facilitating arrestin-3 recruitment [66]. Due to this 22 amino acid substitution, we cannot rule out that the RMOR but not the WT MOR signals to an unidentified effector specific to DORs and that this other effector, rather than arrestin-3 recruitment, prevents tolerance, dependence, and compulsive drug-seeking. However, deletion of the DOR actually reduces tolerance to morphine [67]. If the DOR tail mediated the reduction in tolerance seen in the RMOR through another effector, deletion of the DOR tail would result in increased tolerance, not reduced tolerance. In addition, while morphine is a better analgesic in RMOR mice compared to WT mice, methadone analgesia is unaltered, indicating that it is a change in morphine bias not a global change in signaling that underlies the RMOR morphine phenotypes of reduced tolerance, dependence, and compulsive drug-seeking. Recapitulating “balanced” signaling with exogenous ligands is immensely challenging. Categorizing ligands as ‘balanced’ or ‘biased’ ligands is dependent on accurately

defining both arrestin-3 recruitment and signaling to $G_{i/o/z}$ G protein effectors, both of which contain caveats. For example, while morphine and all its derivates are biased for G protein signaling compared to the endogenous ligands, they do have some ability to engage arrestins. This ability varies depending on the level of GRKs and arrestins. In cells with low GRK expression, efficacy for arrestin recruitment is less than 20% even at saturating ligand concentrations [68] while in cells overexpressing GRKs [69] or arrestin-3 [53], efficacy for arrestin-3 recruitment approaches ~75% compared to DAMGO. This observation has made it difficult to assign a single signaling "bias" value for morphine—though it is always more G biased than the endogenous ligands regardless of GRK/arrestin levels [70–72]. Fentanyl and its derivatives are also G biased, because even though the efficacy of fentanyl for arrestin-3 recruitment is 100% at saturating concentrations, at the EC50 concentration for G protein there is no arrestin-3 recruitment in response to fentanyl [17]. In fact, the only clinically-utilized opioid drug that approaches a signaling bias comparable to endorphins and enkephalins is methadone [73]. None of the handful of "more balanced" tool compounds have been tested *in vivo* because either they have low potency [68], poor solubility [74], or were simply abandoned in favor of "ultra G-biased" ligands.

The past two decades have seen a strong push both preclinically and clinically towards the development of ultra G-biased opioid ligands. This push follows reports that Arr3-KO mice show increased analgesia [35] and reduced tolerance [61] and respiratory depression in response to morphine compared to WT mice, indicating that biased ligands could ameliorate these key side effects. However, several recent reports have failed to reproduce these findings in Arr3-KO mice [50,75]. And clinically, Oliceridine (TRV130), a G protein biased agonist, failed to significantly reduce respiratory depression and was therefore not an improvement over other far less expensive opioids [38]. This has led to a renewed search for drug properties other than G protein bias that could be responsible for the reduced respiratory depressive

effects of ultra G-biased MOR ligands such as TRV130, PZM21 and SR17018. For example, it was recently shown that these 3 compounds have lower intrinsic efficacy for G protein than morphine [41]. If receptors in respiratory centers [76] are more sensitive to changes in intrinsic efficacy than those in analgesic circuits, this observation could explain why the lower efficacy drugs cause less respiratory depression at equi-analgesic doses. However, intrinsic efficacy at G protein is only one of many caveats. New drugs come with many pharmacodynamic changes such as affinity, on and off rate, intrinsic efficacy for G proteins and arrestins, and other potential effectors, as well as differences in pharmacokinetics and engagement with targets other than the MOR. Changes in any of these variables could contribute to the altered effect/side effect profile observed *in vivo,* making it difficult to pinpoint whether signaling bias—or any other property—is paramount.

We posit that any reassessment of the “biased is better” hypothesis should include revisiting the “balanced is better” hypothesis [45]. There are only a handful of drug-like MOR agonists that have been reported to have a balanced signaling profile comparable to the endogenous ligands and none have been tested *in vivo* except methadone. In preclinical models, methadone produces less tolerance and less dependence than morphine [73]. And though it is only rarely used as a first line analgesic in humans because of its highly variable half-life in the population, the few controlled studies that have been done show reduced tolerance to methadone in humans (see review for studies within) [77]. But of course, methadone differs in many aspects of pharmacology from morphine—not just in pharmacokinetics [78], once again hindering the ability to conclude that bias is the defining factor in its reduced side-effect profile. One additional way to examine the role of arrestin-3 recruitment in reducing opioid side effects would be to examine tolerance, dependence, and compulsive drug-seeking to methadone in the MOR 11S/T-A knock-in mouse [44], as methadone would become biased in this mouse model. This would complement the findings from the

RMOR mouse where morphine is now balanced rather than biased.

To date, in silico screens for new opioid ligands have identified only G-biased compounds. This likely reflects the crystal structures used to screen for new ligands. Specifically, it was recently shown using molecular dynamic simulations that the pose(s) displayed by a methadone-occupied MOR are distinct from those for a morphine- or TRV130-occupied- receptor [79]. This indicates that using a different docking structure might identify additional balanced MOR ligands. This is not an implausible goal, as a recent natural products library screen identified a balanced, albeit low potency, MOR agonist [68], suggesting that novel chemical entities could be identified on backbones different from our existing opioids.

In conclusion, here we show that deletion of arrestin-3 does not reduce compulsive drug-seeking in a mouse model of OUD indicating that arrestin-3 does not mediate compulsive drug seeking behavior. As no studies have assessed the abuse liability of the new ultra-biased ligands, our results cast light on clinically relevant risks that should not be ignored. These findings, coupled with recent reports that arrestin-3 deletion does not reduce respiratory depression, indicate that the quest for opioids with reduced side effects should be redirected away from ultra G-biased ligands. Our data also indicate that opioid agonists that provide both analgesia and reward without producing OUD could still be attainable as this is precisely morphine's phenotypic profile in RMOR mice. We currently have no other drugs to treat severe pain, and the opioid epidemic is growing not waning, so this goal remains vital.

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AUTHOR CONTRIBUTIONS

JLW, LF and SG designed the experiments. LF, ZR, JG, AG, MK, and ND performed experiments. Data analysis was performed by LF, SG, RF, KI, IS and JLW. JLW. LF, RF, and IS designed and generated the figures. LF and JLW wrote the manuscript. All authors contributed to manuscript editing.

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Chapter 3

Anxiety state and social status do not predict compulsive drug-seeking be in wild type mice

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INTRODUCTION

The opioid epidemic is a prevailing nationwide public health crisis that has devastated individuals, families, and communities. Approximately 21-29% who receive an opioid prescription will develop an Opioid Use Disorder (OUD) [80]. Predicting which individuals transition from prescribed medical use of opioids to developing an OUD is a complex interaction between environmental factors, drug-induced changes in neurocircuitry, and genetics [81]. Interestingly, in genetically homologous populations of mice, about 30% develop increased drug-seeking behavior, even in a highly regulated environment (see Chapter 2, Fig 2.1E). Here I investigate if increased anxiety and depression in WT mice predict increased drug-seeking behavior. Understanding these underlying risk factors associated with developing an OUD will improve preventative options and treatment outcomes for patients.

Mood disorders, such as anxiety and stress-related disorder (ASRD) and major depressive disorder (MDD) are highly comorbid with opioid use disorders (OUDs) [81]. Persons with major mental health disorders account for 51.4% of the total opioid prescriptions in the United States annually [82]. The directionality of the relationship between mood disorders and OUDs is unclear, but several theories exist: 1) underlying MDD and ASRD are indirect risk factors for the development of OUDS 2) OUDs are causative in the development of a mood disorder 3) both OUDs and mood disorders originate from the same genetic or environmental risk factors.

Mood disorders may serve as an indirect risk for the development of opioids through alterations in emotional regulation surrounding opioid use and abstinence. Anxiety Sensitivity (AS) is the fear of anxiety-related emotions and their potential negative physical, emotional, and cognitive consequences. This is especially relevant during acute withdrawal periods, where physical discomfort during opioid withdrawal syndrome is significant. Fear of experiencing these symptoms can lead people to more frequent relapses to stave off withdrawal symptoms. The presence of AS results in the

increased development of OUDs [83], increased rate of relapse, and is a significant predictor of Addiction Severity [84,85]. Both increased perception of pain and inability of coping with the negative emotions associated with withdrawal predispose people with mood disorders to develop an OUD [85].

The development of an OUD may be causative in the development of mood disorders through the dysregulation of overlapping neuropathways. The mesolimbic dopamine system, which controls reward and motivation, is dysfunctional in both OUDs and mood disorders. Interestingly, upregulation of VTA-NAc is seen in people with substance abuse, while downregulation is seen in MDD. Connections from the NAc to the prefrontal cortex are implicated in executive function and motivational decision making. Upregulation of NAc to the prefrontal cortex are observed in both substance abusers and MDD. The functional connectivity differences observed in depression could predispose persons who begin using opioids to experience more depression and anxiety [86]. Persons with comorbid mood and anxiety disorders exhibit higher rates of relapse, increased economic burden, and greater rates of re-hospitalization following treatment [87].

Inherent differences in personality and social status are another risk factor. When adult mice are cohoused, a social hierarchy emerges where individuals are either dominant or subordinate in their home cage environment [88]. Some studies indicate that lower levels of sociability and increased neuroticism are risk factors for OUDs. Conversely, other studies indicate that increased positive emotionality/extraversion increases motivation and predisposes individuals to be more sensitive to the rewarding effects of opioids [89]. Chronic stress models find that mice of higher social order are more sensitive to defeat than their subordinate counterparts [88]. These individual personality traits of dominant or subordinate mice might have different stress responses to the same stimulus, such as opioid withdrawal.

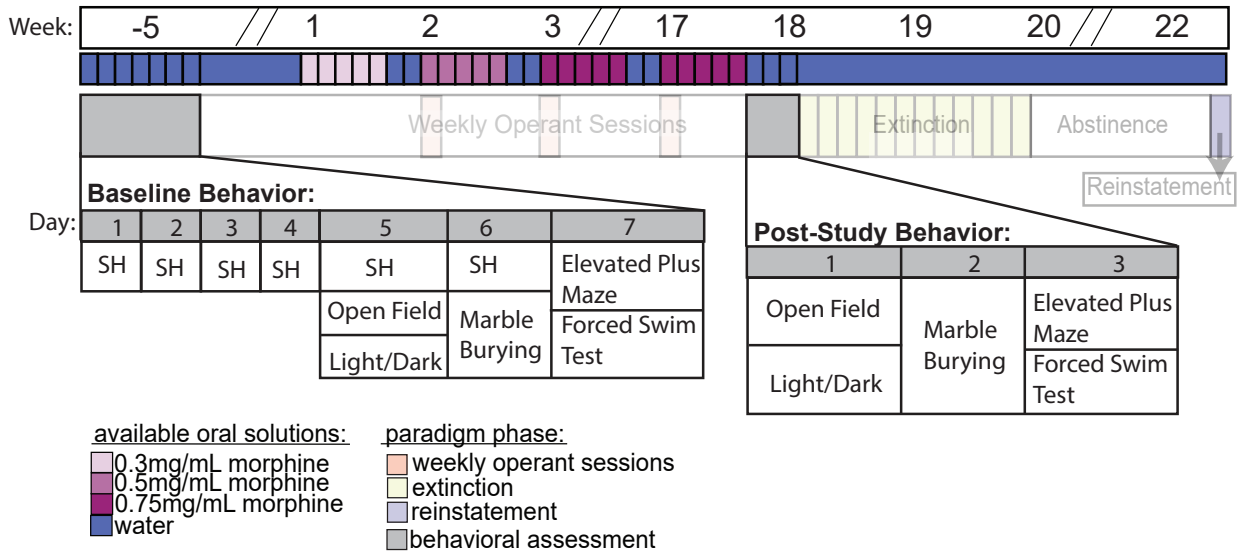

Fig 3.1- Paradigm schematic showing pre- and post-study anxiety assessments in conjunction with a longitudinal oral-operant self-administration paradigm. The top bar shows the week of the paradigm. The middle bar shows oral solutions available to mice during each paradigm phase: water only (blue) and increasing concentrations of morphine sulfate (light pink to dark pink). Gray boxes denote periods in which behavioral assessments took place. Baseline behavior was conducted over 7 days, with days 1-6 measuring social hierarchy status (SH), measurement of Open Field and Light/Dark taking place on day 5, marble burying on day 6, and Elevated Plus Maze/Forced Swim test taking place on day 7. Post-Study behavior was conducted immediately following the last morphine exposure during week 18 and before extinction. Assessments took place over three days, with day 1 measuring Open Field and Light/Dark Transition, day 2 assessing Marble Burying, and day 3 assessing Elevated Plus Maze and Forced Swim.

I have previously shown that a subset of WT mice exhibits increased compulsive drug-seeking behavior in a rodent oral operant self-administration model (Chapter 2). I now seek to determine if the transition of these mice into compulsive drug use can be explained by underlying mood disorders or the development of a mood disorder during opioid exposure. Unlike in humans, assessment of baseline mood and microbiome before drug exposure is possible in murine models. This allows the determination of whether these environmental factors are inherent risk factors, or if morphine induced changes are causative in increased drug-seeking behavior. To accomplish this, I assessed the mood for all mice both before entry into the study (pre-study) and during acute morphine withdrawal (post-study). Understanding the direction of causality

between these key environmental risk factors has broad implications for the prevention and treatment of OUDs.

METHODS

C57BL/6 WT mice (n=26, 19 male, 7 female, 8 bred in-house, and 18 purchased from the Jackson Laboratory Mice) were used for the extent of this study. Mice were assessed for both pre-study anxiety before entering the longitudinal oral operant self-administration study in the 18th week of the paradigm, and post-study during acute morphine withdrawal (See Figure 3.1). Housing conditions and the longitudinal study as described previously (See Chapter 2, methods).

Animal Behavior Assessments:

Forced Swim Test

This protocol is based on Porsolt et al, 1977 [90]. Swim sessions were conducted by placing mice in opaque plastic cylinders (35 cm high x 25 cm in diameter) filled with warm water (26.5°C) to a depth of 20 cm for 6 minutes. A camera mounted above the behavior arena was used to record behavior. Lack of swimming capability was not observed in any of the mice in this study but in the rare event it had, they would have been immediately removed from the swimming apparatus. Immediately following testing, mice were dried off and placed in a holding cage. Mice were allowed to recover for 10 minutes before being returned to the home cage and monitored for signs of hypothermia (lethargy, abnormal breathing) before being returned to the housing rack. During the first two minutes, animals show a high frequency of exploratory and escape-directed behaviors. Each animal is scored for the total immobile time during the last four minutes. Immobility is defined as the absence of movement, except that necessary to keep afloat, including passive floating and turning in the absence of active swimming movement. More time spent immobile is indicative of increased depressive and anxiety-like behavior in this paradigm.

Light/Dark Transition

A well-lit multi-use test arena (Med Associates; Fairfax, VT) with IR arrays on the x and y planes to track the movement of the mouse in a sound-attenuating cubicle was used. For Light/Dark Transition, a dark box insert with a door was placed on one side of the space, occupying 50% of the entire box [91]. Dark box inserts were placed on alternating sides (left/right) to avoid bias. The mouse was placed in the center of the box on the light side, facing the dark side insert door, and was allowed to roam freely for five minutes. The percentage of time spent on the dark side is indicative of increased anxiety-like behavior in this paradigm. Behavior was automatically scored using Medassociates Software.

Open Field

The same multi-use test arena described above was used for the Open Field test. Mice were placed in the center of the arena and left to roam freely for 30 minutes. The amount of time spent in a virtual center zone (25x25cm) in the middle of the arena versus the perimeter was quantified. Increased time spent in the perimeter of the open field box is indicative of increased anxiety-like behavior in this paradigm [92]. Behavior was automatically scored using Medassociates Software.

Elevated Plus Maze

The elevated plus maze apparatus, constructed of wood coated with gloss enamel white paint, is elevated 60 cm above the floor and consists of two open arms (30 cm x 9 cm) and two closed arms (30 cm x 9 cm x 13 cm) that extend from a common central platform (9 cm square). Experimentation takes place in a quiet room. At the beginning of the test, mice are placed on the central platform of the maze facing an open arm and are allowed to freely explore the entire apparatus. The behavior is videotaped for 5 minutes. Time spent in each of the three zone types (open arms, closed arms, and central platform) was analyzed automatically using Noldus EthoVision XT software (Noldus Information Technologies, Leesburg, VA). Increased dwell time in closed arms

is indicative of increased anxiety-like behavior in this paradigm [93].

Marble Burying Task

Mice were placed in a clean cage with one inch of corn cob bedding and 20 glass marbles distributed evenly in a 5x4 grid on the top of the bedding. Marbles were counted as unburied if x>50% was visible, partially buried if 50-90% of the marble was not visible, and totally buried if 90% or more of the marble was not visible. After 30 min, mice were returned to their home cages. The number of buried marbles is indicative of repetitive/compulsive behavior and indicates increased anxiety-like behavior in this paradigm [94].

Assessment of Hierarchical Social Status

Hierarchical social status was assessed using the Social Confirmation Tube test, adapted from Larrieu et all, 2017 [13]. A clear acrylic tube 12 inches in length and 1 ¼ or 1 ½ inches in diameter was used. This diameter is sufficient to allow an adult mouse to pass through the tube, but not turn around or be able to pass its testing partner while in the tube. The 1 ¼ size was used for female mice, while the 1 ½ inch size was used for larger, male mice. Mice were habituated to run through the tube five times a day for two days before the start of the hierarchy test to ensure aversion to passing through the tube did not influence social hierarchy tests. Habituation and testing days took place in a plastic bin, with the acrylic tube tapped to the center. Mice were held by their tails and guided to the center of the tube before being released. The winner of the trial was the mouse that successfully forced its partner to back out of the tube, while it moved forward. Pair-wise interaction for each mouse and all its cage mates was measured using a round-robin tournament style, with each day consisting of three separate trials for each pair. This was repeated for six days consecutively. Testing tubes were cleaned with 70% ethanol when a new pair of animals was tested. The percentage of trials won was divided by the number of predicted trials to be won to account for differences in the number of mice per cage. For example, if a mouse in a cage of four was predicted to

win 25% of trials statistically but actually won 30% of the trials, it would have a "% of expected trials won" of 0.30/0.25=1.2. A score greater than 1.0 indicates that an individual won more trials than expected and is therefore more dominant.

Data Analysis and Statistics

Each individual behavioral assessment (forced swim, light/dark, open field, marbles buried, and elevated plus maze) was normalized using a z-score (z= ((individual response- average response for the population)/s.d. of the population). A composite score was then calculated by summing each of these five individual z-scores for each animal with a larger score indicating increased anxiety. Change in anxiety was calculated for individual measures and composite scores by subtracting the pre-study score from the post-study score (post-study - pre-study). A negative score indicates a decrease in anxiety from post to pre-study, while a positive score indicates an increase from post to pre-study. To assess differences between compulsive and non-compulsive mice, multiple unpaired t-tests with the Holm-Sidak post-hoc correction to account for multiple comparisons was used. Next, to assess if any of the individual behaviors or composite scores covaried with compulsivity scores, a correlation test was run for each individual behavior vs. compulsivity score. Pearson's Correlation Coefficient (r) is reported for each correlation to indicate the strength of the correlation.

RESULTS

Anxiety state is not predictive of compulsive drug-seeking behavior in WT mice

To investigate whether basal anxiety or anxiety produced by opioid withdrawal was predictive of compulsive drug-seeking behavior, I assessed behavior before entrance into the study (pre-study) and during acute withdrawal at the end of morphine exposure post-study) (Fig. 3.1). To do this, I performed a battery of five separate individual behavioral tests: elevated plus maze (EPM), light/dark transition (LDT), forced swim test (FST), marble burying test (MBT), and open field (OF). Data is represented as

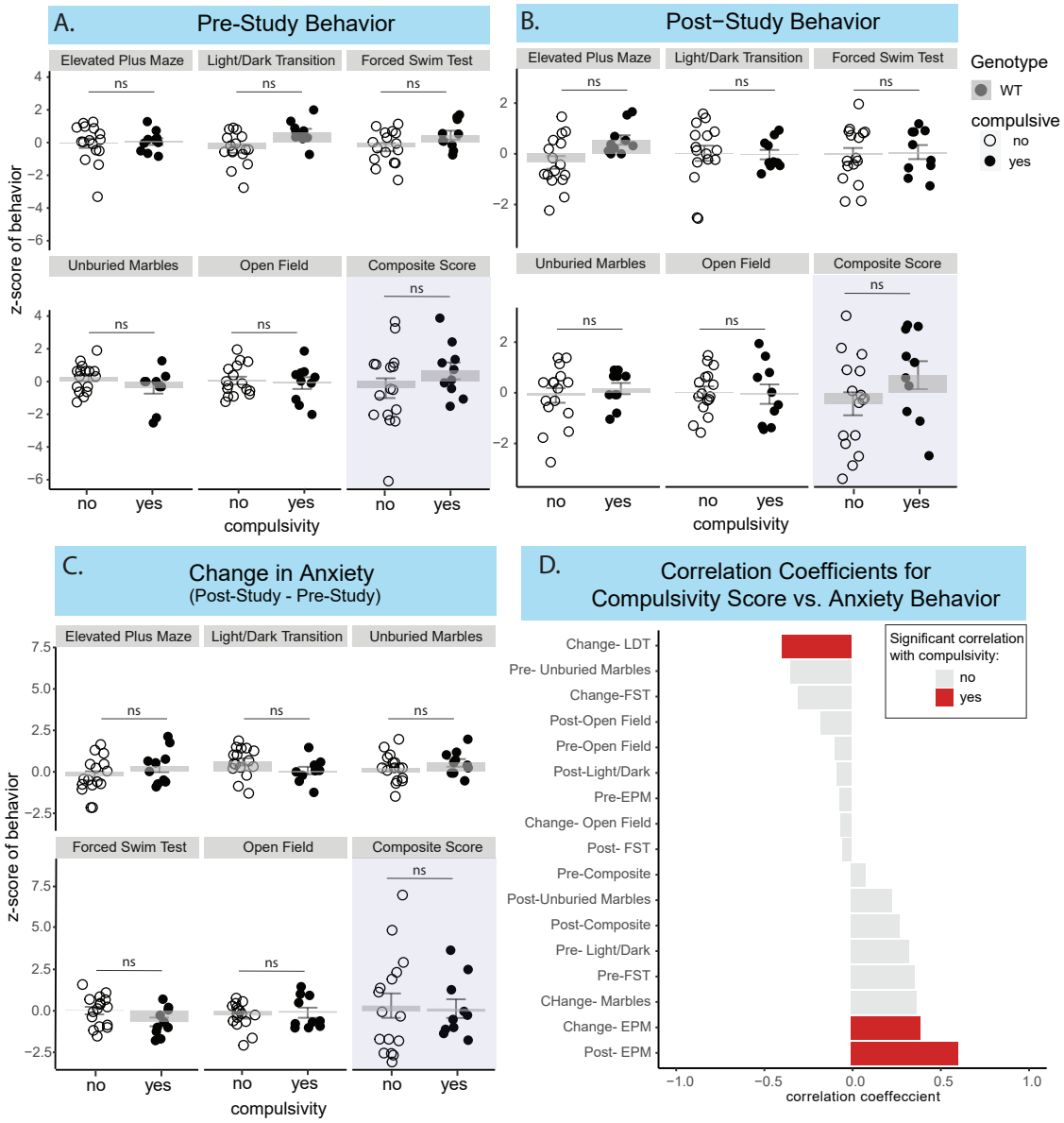

Fig 3.2- Comparison of pre-and post-study anxiety states in compulsive and non-compulsive WT mice. A) Baseline assessment anxiety state in compulsive (filled in circles) versus non-compulsive (open circles) showed no significant differences. B) Post-Study anxiety state showed no significant differences in anxiety state between compulsive and non-compulsive WT mice. C) Change in anxiety state (Post-study behavior- pre-study behavior) showed no significant differences between compulsive and non-compulsive mice. Multiple unpaired t-tests with Holm-Sidak corrections for multiple comparisons were used for A-C. D) Pearson's correlation coefficient from a simple linear regression test for each individual behavioral assessment and each composite score for baseline, post-study, and change in anxiety state (y-axis) versus compulsivity score (x-axis). Significant correlations (red) included change in light-dark transition, change in Elevated Plus maze, and post-study Elevated Plus maze.

the z-score value for each individual test to allow for the cross-comparison of different data types. Each individual measure was collected so that a higher z-score is indicative of increased anxiety (see methods). The five individual assessments were summed into a composite behavioral score (composite score, gray boxes) to encapsulate each individual animal's behavioral state and reduce variability from one individual task. WT mice show no difference in anxiety state when grouped by compulsive (n=10) or non-compulsive (n=16), in pre-study (Fig. 3.2A), post-study (Fig. 3.2B), or change in anxiety (Fig. 3.2C) in composite scores or any of the individual tests. The correlation of individual compulsivity scores, as determined (Fig 2.1, chapter 2), as a numerical variable instead of a categorical variable yielded several significant correlations including change in LDT (r= -0.39), change in EPM (r=0.39), and post-study EPM (r=0.60). Although EPM may be more predictive of compulsive drug-seeking behavior, these data indicate that the anxiety state, as measured by the specific battery of tests in this study, does not correlate with drug-seeking behavior in rodents. This is true for anxiety as an underlying comorbidity (pre-study) and as a response to opioid withdrawal (post-study). Correction of post-study values by subtracting pre-study values (change in anxiety) was similarly not predictive.

Increased dominance in social hierarchy task does not predict compulsive drug-seeking behavior in WT mice

To investigate if natural differences in social hierarchy influence drug-seeking behavior, a social dominance test was conducted six days in a row before entering the study and the fold increase of expected trials was graphed (see methods). Here, a number greater than 1.0 indicates that an individual won more trials than it was projected to win statistically and indicates dominance in the home cage. There was no significant difference between WT mice when grouped by compulsive or non-compulsive (p=0.73, t=0.35) (Fig. 3.3A). No significant correlation was found between social hierarchy and compulsivity in a simple linear regression ((R^2=0.025, p=0.45) (Fig. 3.3B).

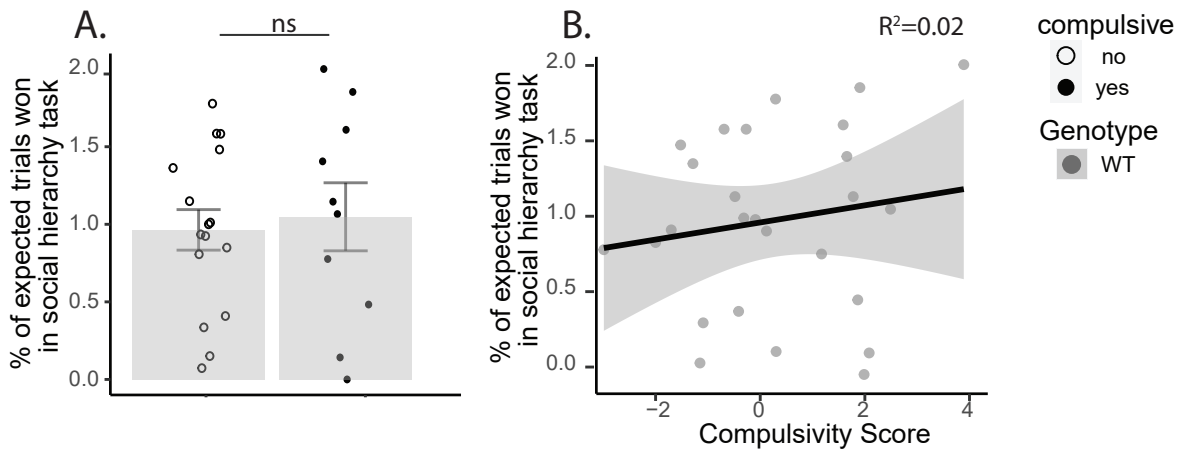

Fig 3.3- Social hierarchy in compulsive and non-compulsive WT mice. A) Social hierarchy does not differ between compulsive (filled in circles) vs. non-compulsive (open circles) WT mice (students t-test). Y-axis represents % of expected trials won. See methods for a detailed explanation. B) Correlation of social hierarchy and compulsivity score in a simple linear regression (R^2=0.025, p=0.45).

DISCUSSION

In this study, I demonstrate that pre-study basal anxiety and post-study morphine withdrawal induced anxiety states are not significantly different in compulsive drug-seeking mice. Therefore, it is not possible to determine if anxiety disorders serve as a predisposition for developing OUDs or if anxiety disorders are a byproduct of drug use. These results are surprising, considering that the commodity of anxiety, depression, and other related mood disorders are well established in human populations [85,91,99–101]. These results may highlight a limitation in assessing anxiety in a rodent population. Tests such as the forced swim and open field tests were originally designed to test the efficacy of anxiolytic and antidepressant drugs and not natural mouse behavior [102,103]. C56BL/6 WT mice are the gold standard for assessing behavioral phenotypes in a rodent model, but even in WT mice, the tests can exhibit variability and lack of reproducibility. For example, when administered chlordiazepoxide, a common anxiolytic agent, C57BL/6 mice exhibited no reduction in anxiety-like behavior in open field or light-dark test [104]. This demonstrates that many of the tests used to measure

rodent anxiety may not provide direct insight into human conditions that are improved by anxiolytic medications, a sediment echoed by others in the field [105,106].

Therefore, a major limitation of this study is persistent controversy exists on the validity of assessing rodent anxiety using classical tests. Day-to-day variability can be a result of local conditions not perceivable by human senses, such as smell and noise levels, experimenter mood, estrus cycles in female rodents, and many other variables [107,108]. The lack of controls limits the comparison of groups run at different points in time. One possibility would be to run WT mice with anti-anxiolytic drugs at each assessment to determine the baseline response for each day and standardize by day. However, as mentioned, even behavioral responses to anxiolytic drugs can be variable. In addition to lack of reproducibility, stress responses in animals that produce anxiety related behaviors are also fundamentally different than in humans [109,110]. Fear and anxiety in a rodent promote increased vigilance, freezing, and elevated heart rate [104]. These may result in less exploratory behavior of the environment, like in an elevated plus maze. However, it could also result in freezing in place, regardless of positioning in an open or closed arm of the elevated plus maze. Many of these tests are perhaps ethologically relevant to rodents but fall short in comparison to the complexity of human mood disorders. A lack of translation of rodent ‘anxiety’ behavior into human anxiety remains a pivotal issue to overcome for modeling mood disorders in rodents.

Chapter 4

Evaluation of the interaction of anxiety state and compulsive drug-seeking behavior in multiple transgenic mouse lines

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INTRODUCTION

Although none of the environmental risk factors were predictive of compulsive drug seeking in WT animals, it is possible the anxiety state varies by genotype, or that the anxiety state is more predictive of compulsive behavior in Arr3-KO mice. To investigate this, I next determine if pre- or post-study anxiety measures vary by genotype as baseline anxiety state has yet to be assessed in both RMOR and Arr3-KO mice. Many transgenic mouse models have been shown to have altered anxiety, including Beta-arrestin-1 KO mice showing reduced anxiety in Light Dark Transition tests [117], but arrestin-3 knockout mice remain to be determined. As arrestin-3 has been shown to bind >800 GPCRS [118] and act as scaffolding proteins for multiple signal transduction pathways, it is possible the lack of Arr3-KO could alter the baseline anxiety state. I then investigate how anxiety or social hierarchy co-vary with compulsive drug-seeking in Arr3-KO mice separately to determine if the environmental factors in this study predisposed these mice to develop compulsive drug-seeking behavior. Methods and data analysis are identical to Chapter 3.

METHODS

Mice

The mice in this chapter are identical to those used in Chapter 2. See Chapter 3 for a timeline of anxiety measures.

Data analysis

Differences between genotypes (WT, RMOR, Arr3-KO) were first determined by ordinary one-day ANOVA then Tukey's multiple comparisons test to determine which genotypes varied. All p-values reported for genotype differences are adjusted p-values. To assess differences between compulsive and non-compulsive in Arr3-KO, multiple unpaired t-tests with the Holm-Sidak post-hoc correction to account for multiple comparisons. Next, to assess if any of the individual behaviors or composite scores covaried with

compulsivity scores, a simple correlation test was run for each individual behavior vs. compulsivity score. Pearson's Correlation Coefficient (r) is reported for each correlation to indicate the strength of the correlation. An unpaired, two-tailed t-test was used to determine differences between compulsive and non-compulsive mice in the social hierarchy task.

RESULTS

Pre-study anxiety is higher in RMOR and Arr3-KO mice compared to WT

Anxiety scores varied by genotype in baseline measurements (Fig. 4.1, p=0.0001, f=12.66) with Arr3-KO and RMOR exhibiting increased baseline anxiety composite scores compared to WT (adjusted p values: p=0.0041 for WT vs RMOR, p<0.0001 for WT vs Arr3-KO) as well as several statistically significant differences in individual measures. No difference in EPM (p=0.27, f=1.333) or marble burying was seen across genotypes (p=0.12, f=2.16), but increased anxiety in LDT and OF was seen in RMORs compared to WT mice (adjusted p-value: p=0.0002, p=0.0498). Arr3-KO showed increased anxiety compared to WT mice in FST (p= 0.02), with many other values trending towards significance such as OF (p=0.051) and marble burying (p=0.098). Taken together, these data suggest that basal anxiety state does differ between mice of WT, RMOR, and Arr3-KO genotypes, although significant variability between tests exists.

Post-study anxiety is higher in Arr3-KO mice compared to WT

Post-Study anxiety also varied by genotype (Fig 4.2, p=0.050, F=3.144) with Arr3-KO mice showing increased anxiety as compared to their WT (p=0.04). There was no difference between genotypes in LDT (p= 0.24, F=1.42), or FST (p= 0.94, F=0.05). EPM was significantly higher in Arr3-KO compared to WT (p=0.049) and OF was trending towards significance (p=0.051). Although several RMOR vs WT comparisons were significant, they did not vary in the same direction. RMOR mice exhibited significantly

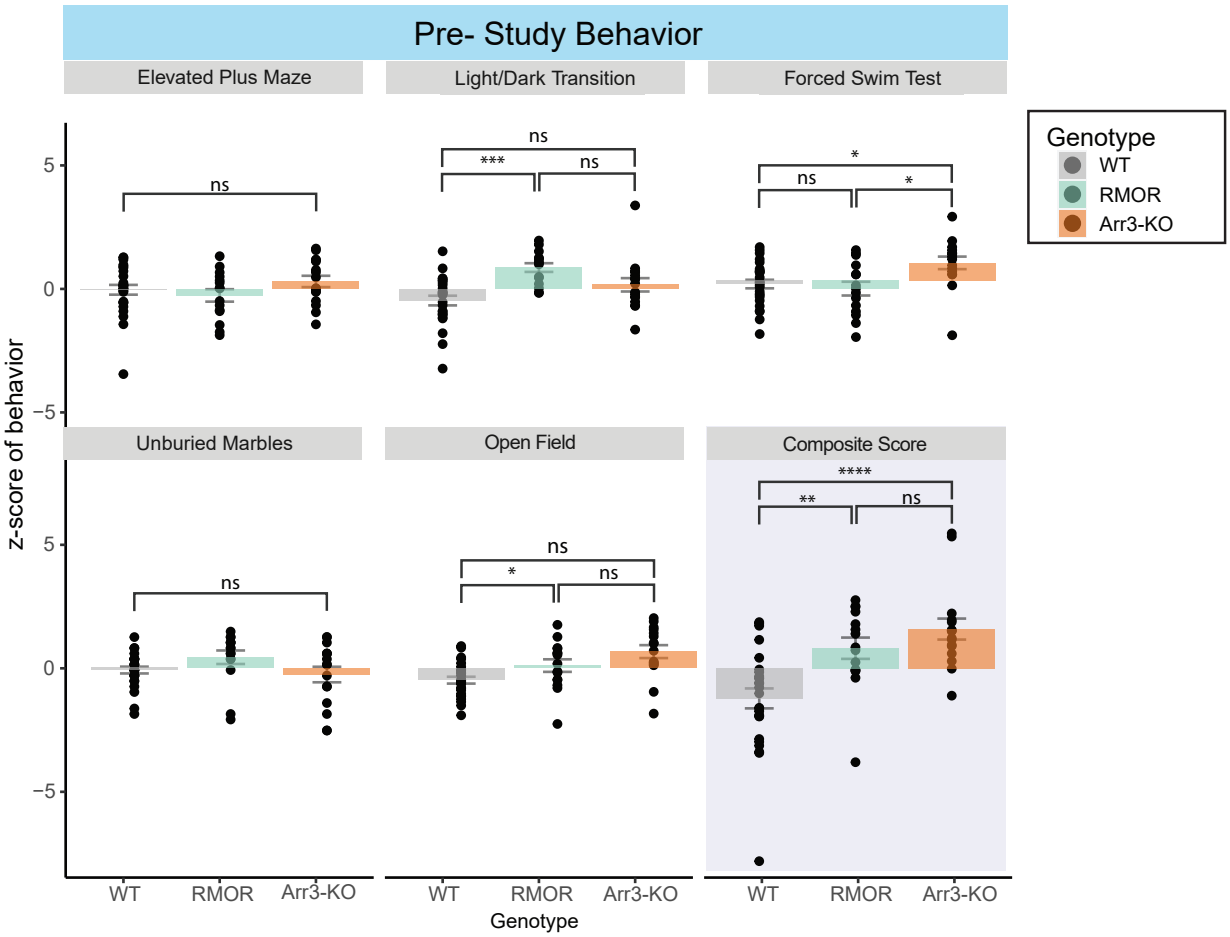

Fig 4.1- Pre-study anxiety in WT, RMOR, and Arr3-KO mice. Baseline behavior by z-score of each individual task (Elevated Plus Maze, Light/Dark Transition, Forced Swim test, Marble Burying task, Open Field) for WT (gray), RMOR (teal), and Arr3-KO (orange). The composite score (gray box) shows the sum of the five individual z-scores for each individual. Differences in each behavioral measure and between composite scores were assessed by a one-way ANOVA with Tukey's multiple comparisons. Filled in circes represent all mice, compulsive and non-compulsive.

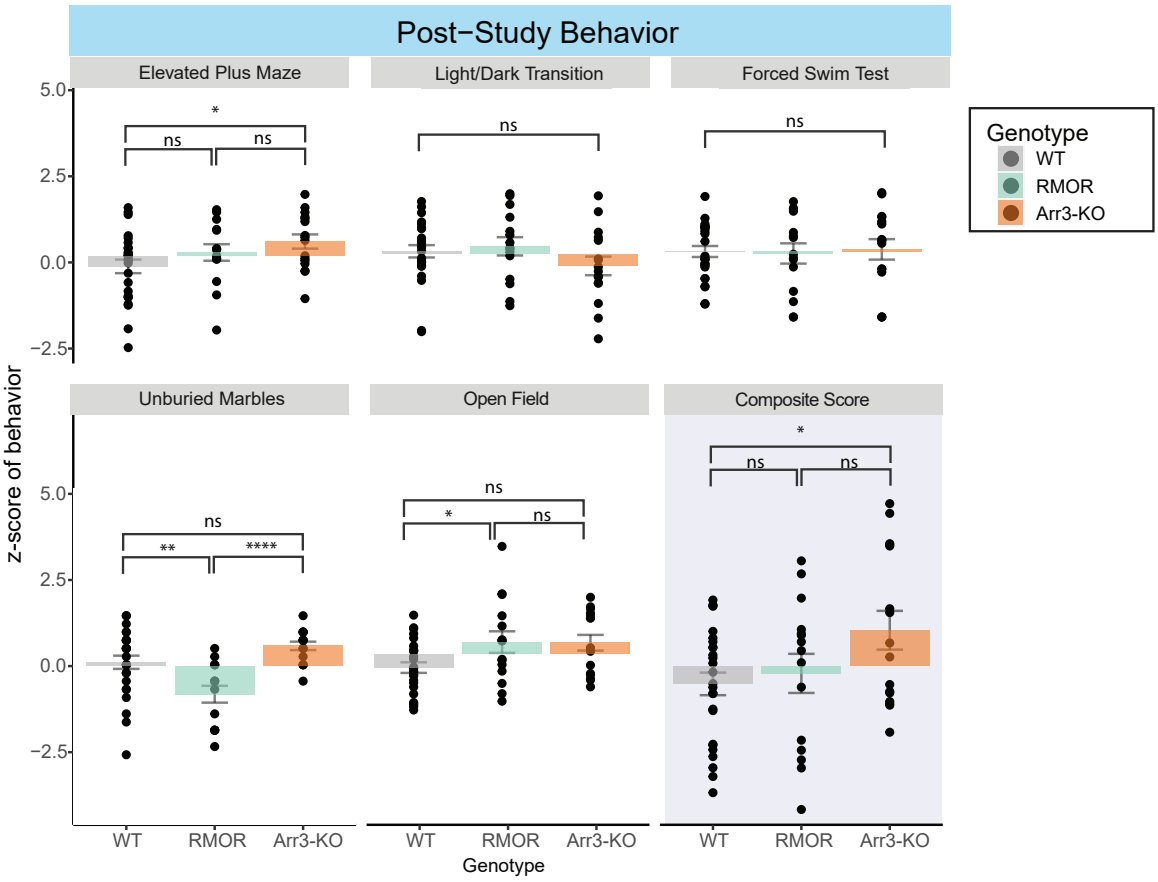

Fig 4.2- Post-study anxiety in WT, RMOR, and Arr3-KO mice. Post-Study anxiety by z-score of each individual task and for composite scores for WT (gray), RMOR (teal), and Arr3-KO (orange). Differences in each behavioral measure and composite scores were assessed by a one-way ANOVA. Filled in circes represent all mice, compulsive and non-compulsive.

less marble-burying behavior (p=0.0046) but significantly more anxiety-like behavior in the OF test (p=0.049). Overall, a difference in post-study anxiety was observed between genotypes indicating that withdrawal from morphine can produce variable amounts of anxiety in a mouse model.

Anxiety state is not predictive of compulsive drug seeking in Arr3-KO mice. Similarly in Arr3-KO mice, neither pre-study (Fig 4.3A), post-study (Fig 4.3B), nor change in anxiety (Fig 4.3C) showed any significant differences between non-compulsive (n=10) and compulsive (n=6) mice in composite scores or any individual scores. No significant correlations were found between numerical compulsivity scores and any of the behaviors observed (Fig 4.4D).

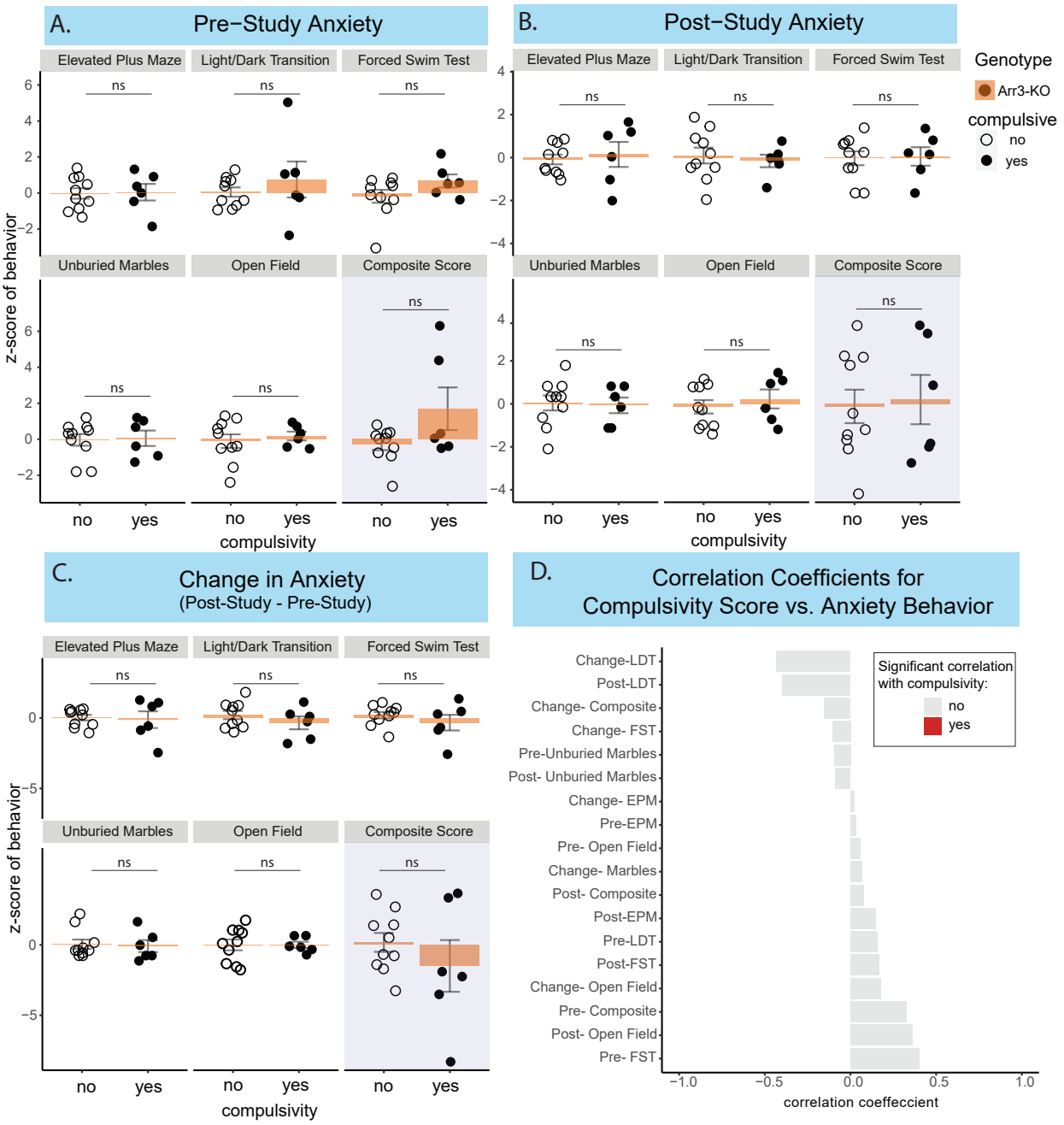

Fig 4.3 – Pre-study, Post-study, and change in anxiety in compulsive and non-compulsive Arr3-KO mice. A) Baseline assessment anxiety state in compulsive (filled in circles) versus non-compulsive (open black circles) showed no significant differences. B) Post-Study anxiety state showed no significant differences in anxiety state between compulsive and non-compulsive WT mice. C) Change in anxiety state (Post-study behavior- baseline behavior) showed no significant differences between compulsive and non-compulsive mice. Multiple unpaired t-tests with Holm-Sidak corrections for multiple comparisons were used for A-C. D) Pearson's correlation coefficient from a simple linear regression test for each behavioral assessment and each composite score for baseline, post-study, and change in anxiety state (y-axis) versus compulsivity score (x-axis). No significant correlations were found.

Social dominance is predictive of compulsive drug-seeking behavior in Arr3-KO mice.

To investigate if natural differences in social hierarchy influence drug-seeking behavior, a social dominance test was conducted six days in a row before entering the study and the fold increase of expected trials was graphed (see methods for more details). Here, a number greater than 1.0 indicates that an individual won more trials than it was projected to win statistically and indicates dominance in the home cage. A significant difference between compulsive and non-compulsive Arr3-KO mice (p=0.039, t=2.28) was observed (4.4A). Correlation between the social hierarchy score and compulsivity score was not significant (R^2=0.08, p=0.28) (4.4B).

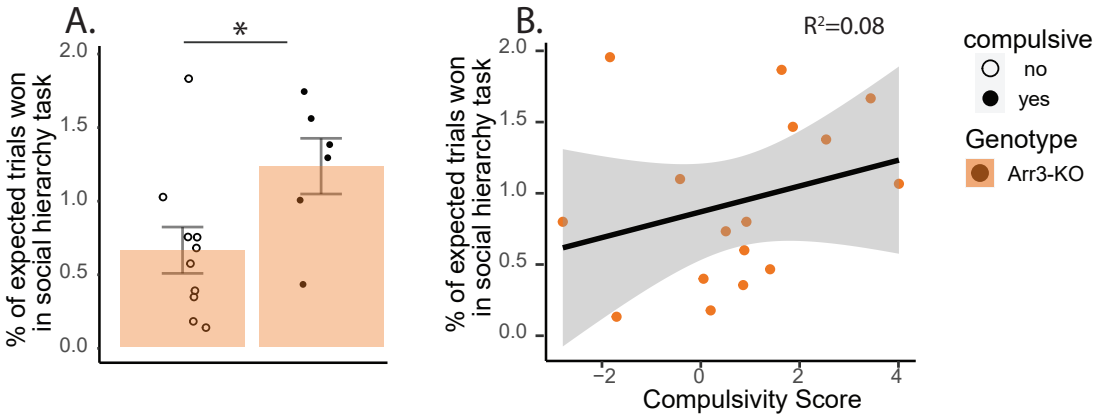

Fig 4.4- Social hierarchy in compulsive and non-compulsive Arr3-KO mice. A) Social hierarchy is significantly different between compulsive (filled in circles) vs. non-compulsive (open circles) Arr3-KO mice (students t-test, p=0.039, t=2.28). Y-axis represents % of expected trials won. See methods for a detailed explanation. B) Correlation of social hierarchy and compulsivity score in a simple linear regression (R^2=0.08, p=0.28).

DISCUSSION

This study demonstrates that Arr3-KO mice exhibit increased anxiety compared to WT mice in both pre-study and post-study measurements. RMOR mice exhibited increased pre-study anxiety, but not post-study anxiety (Fig. 4.1, Fig. .2). Interestingly, although RMOR mice exhibit increased anxiety in pre-study measurements, no RMORS exhibited compulsive drug-seeking behavior (Fig. 2.1E). Additionally, WT and Arr3-KO were indistinguishable in their compulsive drug-seeking profiles (Fig. 2.1E), but Arr3-KO mice exhibited both increased pre-study and post-study anxiety as compared to WT. Anxiety state, either pre-study, post-study, or change in anxiety, was not predictive of compulsive drug-seeking behavior in Arr3-KO mice (Fig. 4.3). However, social hierarchy was predicative (Fig 4.4). These data suggest that the anxiety state is not predictive of compulsive drug-seeking behavior in either WT or Arr3-KO. However, there is a significant difference in anxiety between genotypes- with Arr3-KO mice exhibiting increased anxiety.

This study does have limitations. Namely, the anxiety data was collected at different points in time, spanning over a year. The differences in anxiety state could be attributed to the genotypes being run on different days. Standard practice for animal behavior would be to run all genotypes on the same day or to run multiple groups of mixed genotypes to correct for minute details that may affect animal behavior (See Chapter 3 discussion for details). Therefore, the next logical step for this study would be to run WT and Arr3-KO mice in mixed genotype groups on at least three separate days to determine if the increase in Arr3-KO mice exhibited here is reproducible.
Overall, differences in anxiety state by strain of mouse are well documented and are an important consideration when running a study of several genotypes [113,114]. The anxiety state could have affected drug-seeking behavior by preventing mice from engaging in drug-seeking behavior because of a freezing response, or it could have increased drug-seeking behavior by encouraging self-mediating behavior to relieve

anxiety symptoms. Deletion of Arr3 could alter the regulation of many GPCRs, such as serotonin receptors, and affect basal anxiety state [115–118]. These hypothetical alterations would not be attributable to alterations in drug-seeking behavior, but rather an underlying characteristic of the genotype itself. Therefore, evidence that RMOR mice have higher pre-study anxiety but do not exhibit drug-seeking behavior provides further evidence that the differences in drug-seeking behavior seen are due to altered signaling in Arr3, not due to an artifact of the genotype itself. Although rodent models of anxiety inform the development of improved pharmaceuticals and contribute to understanding the biological basis of anxiety, they certainly have limitations especially in mice [119]. Rodents and humans differ significantly in the ability to engage in complex thinking that is not captured using rodent models, especially when considering the complexity of OUDs and associated comorbidities [105,106,120].

Chapter 5

Morphine induced changes in gut microbiome composition yield biomarkers of compulsive drug-seeking behavior in WT mice

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INTRODUCTION

The ability of the human gut microbiome to modulate behavior and affect disease severity has been a growing area of research in recent years. The enteric and central nervous systems (ENS, CNS) are intricately connected, exhibiting substantial bidirectional physical and chemical communication. The vagus nerve physically connects the CNS and ENS and is capable of carrying information perceived in the ENS to the forebrain and midbrain [101], including modulating pain perception [102]. Chemical communication occurs through neurotransmitters, hormones, signaling molecules generated by intestinal microbiota, and activation of immune cells [26,101,103]. The homeostasis of this communication is upheld by the diverse gut microbiome. However, this homeostasis is perturbed in multiple disease states including many metabolic diseases, such as type two diabetes and obesity [104]. More surprisingly, there is now significant evidence that the ENS modulates neuropsychiatric disorders such as autism [105], depression [106], Parkinson's disease [107], and addiction-related behavior [108].

Mu-Opioid receptors in the ENS inhibit the contraction of the smooth muscle, leading to reduced gastrointestinal motility. This leads to chronic constipation as well significant changes in the microbiome composition. Morphine induces dysbiosis- a disruption in the healthy gut microbiome's homeostasis characterized by diversity loss (Fig. 5.1, Aii) [31,108]. This leads to increases in pathogenic bacterial community members associated with increased epithelial permeability and translocation of bacteria, leading to systemic inflammation (Fig. 5.1, Ai) [109]. Previous research shows that the microbiome plays an essential role in regulating reward in response to drugs of abuse [110]. However, the exact mechanisms and species responsible for these changes are undetermined. The human microbiome is home to trillions of microorganisms, and individuals' microbiomes vary greatly. In addition to inherent individual variation, opioids induce profound changes in the microbiome composition. Therefore, it is possible that

individual microbiome composition, either before morphine treatment or in response to morphine treatment, may underlie some of the individual variability seen in drug-seeking behavior.

Given the substantial interaction of the CNS and ENS and previous literature suggesting the role of the microbiome in behavior modulation, I propose the microbiome may serve as another environmental risk in the development of compulsive drug-seeking behavior in genetically homologous mice. Here I seek to determine if initial microbiome composition or changes in microbiome composition in response to morphine treatment are predictive of which individual WT mice develop highly compulsive drug-seeking behavior. To do this, I collected fecal samples throughout the longitudinal oral operant self-administration paradigm and used 16S sequencing to characterize biomarkers of compulsive both pre-, mid-, and post-morphine treatment. I hypothesize that there will be several biomarkers that are unique to both non-compulsive and compulsive mice.

METHODS

Mice

One cohort of C57/B6 wildtype (WT) mice ordered directly from The Jackson Laboratory (n=16, male, 8 weeks old) were used for this experiment. These mice are a subset of the WT mice that were run through the same oral operant self-administration paradigm outlined in Chapter 2. Mice were group housed until entry into the two-bottle choice phase of the paradigm where they were singly housed to accurately record individual morphine consumption. Extra enrichment in the form of BioServ running wheels was provided to compensate for long-term single-housing conditions. All mice were 12 weeks old at the start of operant training. Mice were housed on a reverse 12-hour light/dark cycle (light out at 8 AM, on 8 PM) for the extent of the study so that all behavioral

tasks were performed during the active cycle. Mice had access to food and water *ad libidum*.

16S metagenomic sequencing of rodent fecal samples

To investigate whether gut microbiota co-varies with compulsive drug-seeking behavior, fecal samples from throughout the paradigm were collected with a sterile technique and sent to the laboratory of Dr. Cheryl Whistler (University of New Hampshire). They performed 16S sequencing on each sample indicated by a bubble in Figure 5.1B. In short, amplicon libraries were generated from the V4-V5 variable regions following Earth Microbiome Project protocols and best practices [111]. Pooled amplicon libraries were sent for paired-end (250 bp x 2) sequencing on the Illumina NovaSeq600 at the Hubbard Center for Genome Studies at the University of New Hampshire. Demultiplexes reads were sent through a reproducible pipeline (DADA2) in combination with custom scripts for quality control. Taxonomy was subsequently assigned using the GreenGenes reference database [112]. Corncob regression models were used to identify community members that were significantly different in abundance between compulsive and non-compulsive mice [113]. Linear discrimination analysis of effect size (LEfSe) was then used to identify biomarkers that most likely explained differences between compulsive and non-compulsive mice [114].

RESULTS

Chronic morphine alters the gut microbiome

Morphine-induced changes in the gut microbiome are well documented [31,32,103,108] but are usually observed as a population. To monitor individual variations in microbiome composition, I took paired fecal samples during chronic morphine exposure (Fig. 5.1B). The temporal changes in gut microbiome in response to morphine are shown in Figure 5.1C. Pre-morphine samples (peach) cluster together but exhibit individual variability. Early, mid, and late-morphine groups show a gradual shift in

microbiome composition that does not rebound to pre-morphine composition. Like pre-morphine conditions, individual variability in the response to chronic morphine treatment was also observed (Fig. 5.1C).

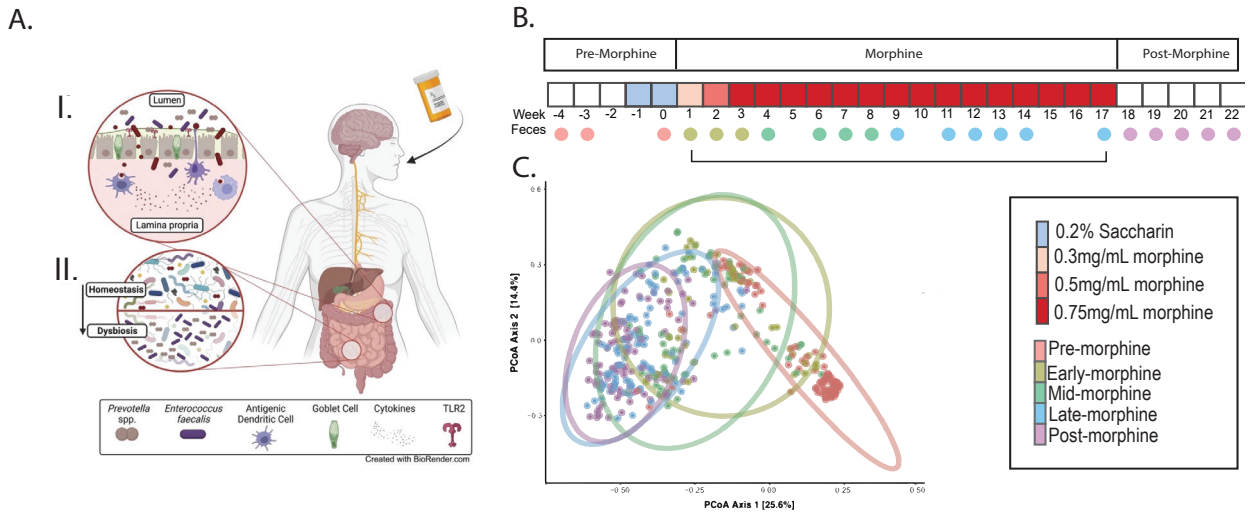

Figure 5.1 – The effect of chronic morphine on the microbiome composition of WT mice. A) I) Chronic morphine results in increased translocation of harmful pathobiont bacterial species, leading to cytokine release and activation of microglia. II)) Collapse of community diversity in the gut microbiome resulting in a shift from homeostasis to dysbiosis. B) Paradigm timeline describing the collection of fecal samples by the week before morphine (light peach), early-morphine (yellow-green), mid-morphine (green), late-morphine (blue) and post-morphine (purple). Middle, colored bars show available oral solutions, in addition to water: 0.2% saccharin (light blue) before week 0, 0.3mg/mL morphine (pale pink) in week one, 0.5 mg/mL morphine in week two, and 0.75 mg/mL morphine from week 3-17. C) Temporal changes in the gut microbiota diversity assessed by Bray-Curtis dissimilarity were analyzed by principal coordinates analysis (PCoA) shows that microbiome community members change over time with morphine exposure and do not rebound to starting composition after morphine was removed.

Identification of bacterial biomarkers for compulsive drug-seeking behavior

Fecal samples were grouped in pre-morphine (peach bubbles, n=3), morphine (yellow-green, green, and light blue bubbles, n=13), and post-morphine (purple bubbles, n=5) (Fig. 5.1B). Mice were categorized as compulsive or non-compulsive by their

compulsivity scores, as determined in Chapter 2. A comparison of compulsive and non-compulsive mice yielded several biomarkers that were significantly different between these populations. Poppy symbols indicate bacterial species that were higher in compulsive mice, while asterisks indicate bacterial species that were higher in non-compulsive mice (5.2A).

Previous studies have shown that morphine increases the relative abundance of pathogenic bacteria such as *Avobacterium, Enterococcus, Fusobacterium, Sutterella, Clostridium, Rikenellaceae, and Ruminococcus* [108]. Most of these species were not significantly different between compulsive mice and non-compulsive mice. However, two species of *Ruminococcus* were significantly higher in non-compulsive mice and *Bacteroides* were higher in non-compulsive mice. Previous literature indicates *Bacteroides* co-varies with increasing MOR receptor expression in the colon [115]. This may indicate counter-adaptation in response to chronic morphine that was not present in non-compulsive mice. Differences in commonly protective bacteria such as *lactobacillu*s were not observed to be higher in non-compulsive mice. Further analysis into these species-specific alterations in relative abundance and their potential significance in predicting compulsive drug-seeking behavior is in progress (Fig. 5.2A).

Proposed model of the modulation of drug-seeking behavior by gut microbiota

Morphine acts on MOR in the ENS to slow gut motility and induce constipation. Chronic morphine perturbs morphine homeostasis in the gut by increasing pathogenic bacteria (*Enterococcaceae*, and *Prevotella* as examples) *(Fig 5.2B II)*. Morphine also decreases beneficial bacteria such as *Lactobacillus* that control the growth of pathogenic bacteria. Left unchecked, these changes result in a collapse of the microbiome and reduced diversity *(Fig 5.2B II)*. These pathogenic bacteria activate Tol-like receptor (TLR) mediated bacterial translocation across the gut, leading to further inflammation and immune response *(Fig 5.2B III)*. Activation of microglia and astrocytes signifies

the immune system response into a pro-inflammatory state, marked by the release of cytokines. In the brain, microglial cells are activated and promote subsequent neuroinflammation, compromising the blood brain barrier. *(Fig 5.2B I)*.

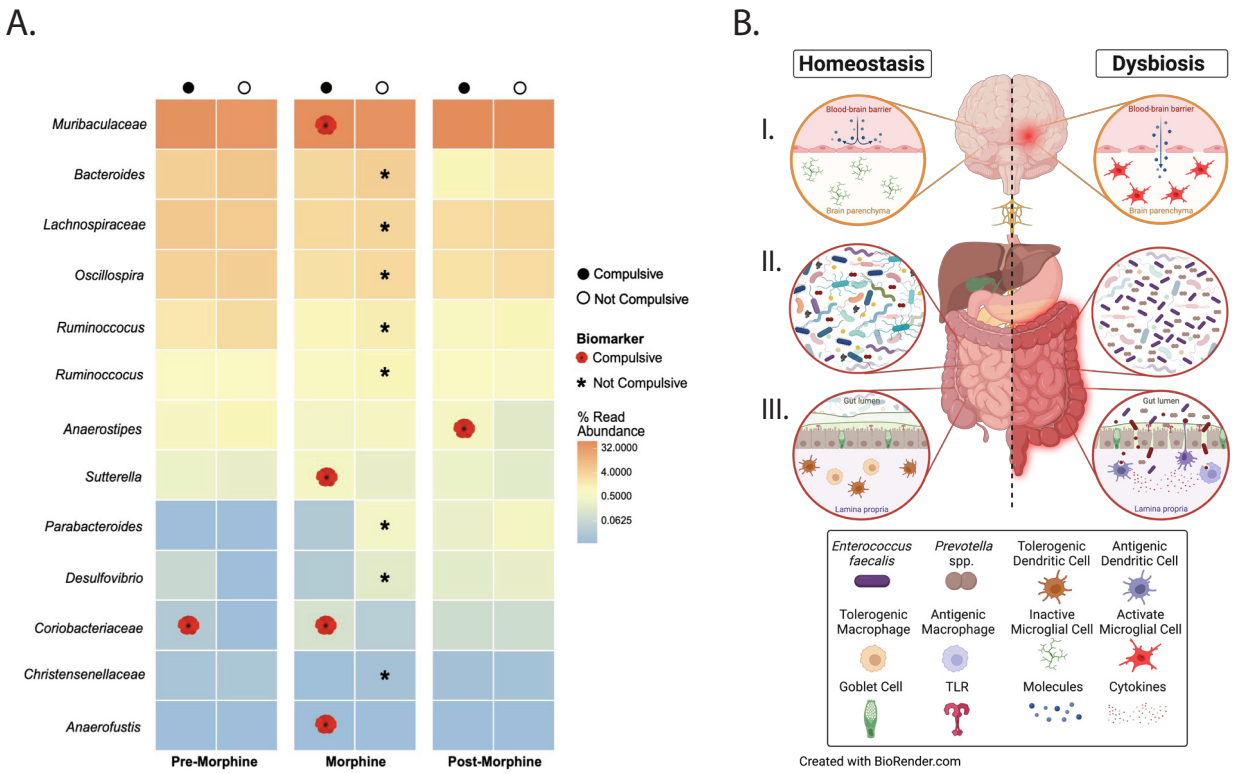

Figure 5.2- Biomarkers of compulsive drug-seeking behavior and proposed model of drug-seeking behavior modulation by gut microbiota. A) Compositional changes in the gut microbiota that distinguish compulsive mice from non-compulsive mice. The percent read abundance was calculated by dividing the number of sequencing reads for individual community members by the total number of sequencing reads. Biomarkers of compulsivity were then identified among community members whose abundance was significantly altered in compulsive versus non-compulsive mice. See methods for details on analysis. B) Proposed model of how changes in gut microbiota can result in a shift from homeostasis to dysbiosis in the brain (I), in the gut (II), and by promoting TLR initiated translocation of pathobiontic bacteria such as *Enterococcus faecalis* and *Prevotella* across the gut epithelium (III).

DISCUSSION

Here I demonstrated that the rodent microbiome is profoundly altered in WT mice in response to chronic morphine treatment and that these changes are not reversible within one month (Fig. 5.1C). I then show that the microbiome of compulsive and non-mice have several bacterial species that are differentially altered in response to morphine treatment (Fig. 5.2A). These bacterial species could serve as biomarkers for identifying individuals who have an increased propensity to develop an OUD. I propose a model that gut dysbiosis results in immune activation and systemic inflammation leading to an increase in permeability at both the gut epithelium and the blood brain barrier (Fig. 5.2B) [121, 123, 125]. There are several potential outcomes that could underlie drug-seeking behavior: I) Increased blood-brain barrier permeability could lead to neuroinflammation and the manifestation of chronic pain, increasing demand for opioids [37]. II) Higher plasma levels of morphine across the leaky blood brain barrier results in increased morphine reward. III) altered pharmacokinetics of morphine-3-glucuronide metabolism by deconjugating microbes in the gut, leading to reduced reabsorption of morphine and higher plasma consumption [128].

A portion of our findings replicated previous findings, but several data points are puzzling. Overall, *Rumminococcus* does decrease between pre- and late morphine, but it was significantly higher in non-compulsive mice. This is unexpected, as *Rumminococcus* is a pathobiont bacteria and one may think it would be higher in *compulsive* mice [129]. *Bacteroides* were higher in non-compulsive mice and previous literature indicates *Bacteroides* co-varies with increasing MOR receptor expression in the colon. This could indicate that non-compulsive mice undergo counter adaptations, like increased MOR expression [128]. Perhaps this could serve as a protective mechanism to counteract desensitized morphine receptors. Similarly, it is unexpected that beneficial bacteria such as *Lactobacillu*s are not increased in non-compulsive mice compared to compulsive. This is also unexpected, as *Lactobacillus* has been shown

to have a role in the prevention of obesity, diabetes, and inflammatory bowel disease [135,136].

This study presents several limitations. Limited sample size in compulsive (n=4) presents one such limitation. It is possible that new biomarkers would immerge in a larger sample size, or that those identified in this study would not be upheld. Another limitation is that differences in experimental design or length of morphine exposure make cross comparison of multiple studies difficult. This study is substantially longer than other studies, such as Zhang et al [128] where they identify changes after 6 days of morphine treatment. Additionally, a major caveat of 16S sequencing is that the species level information is not available. Therefore, the *rumminococcus* species characterized in our study could be a different strain than previously identified *rumminococcus* species.

The identification of specific bacterial species that reliably modulate the development of Opioid Use Disorder (OUD) in a human population is still a long way off. However, our initial findings indicate that there are differences worth integrating further. Identification of these species would have far-reaching implications for the treatment and prevention of OUDs. Identification of a protective bacterial species could lead to the development of a probiotic that counteracts the dysbiosis enacted by chronic morphine. Prevention of dysbiosis could result in the integrity of the gut and brain epithelium being preserved, reducing neuroinflammation and potentially the development of an OUD [136]. Similarly, the identification of pathobiont species that predisposes a person to increased drug-seeking behavior could spur the development of antibiotics or the formulation of pro-biotics that specifically counteract these changes. Supplementation with beneficial probiotics reduce the development of OUDs for some people, therefore mitigating the proportion of people transition to drug use [133]. Further research is needed to validate these ideas, but the gut-brain connection remains a promising area of research for the prevention of OUDs.

Chapter 6

Conclusions and Future Directions

I) Restatement of conclusions and implications

Collectively, the experiments in this dissertation provide experimental evidence aimed to improve opioid pharmaceutical use by 1) providing information on the ideal cellular signaling profile and 2) describing how anxiety, social hierarchy, and microbiome composition could predict the transition of individuals to use drugs compulsively. In Chapter 2, I demonstrated that WT and Arr3-KO mice exhibited the same degree of compulsive drug-seeking behavior as determined by a composite compulsivity score. Conversely, RMOR mice exhibited a reduction in drug-seeking behavior. This suggests that Arr3 does not mediate drug-seeking behavior. In fact, these data suggest that the recruitment of Arr3 and subsequent internalization of the receptor plays a protective role in the development of compulsive drug-seeking behavior. These results answer a long-standing question in the field concerning the role of Arr3 in mediating the negative side effects of opioid pharmaceuticals. Previous research suggested that Arr3 mediated other negative side effects, such as analgesic tolerance and respiratory suppression [41,66]. However, the validity of these findings has been questioned when multiple research groups have been unable to replicate them [55,79]. The data presented in this dissertation suggest that the development of new opioid pharmaceutical that causes robust Arr3 recruitment like endogenous ligand signaling (balanced agonism) could improve the development of OUD in a human population.

In Chapters 3-5, I investigate the validity of using individual predispositions in anxiety, social status, and microbiome to predict the transition to compulsive drug-seeking. In Chapter 3, I investigate how underlying anxiety state or anxiety in response to morphine withdrawal is predictive of which individuals in a WT population transition to

compulsive behavior. I found that neither pre-, post-, or change in anxiety was predictive of which mice developed compulsive drug use. Ranked social status was similarly not predictive in WT mice. This implies that a mouse model of anxiety and depression may be inadequate to assess the anxiety state, as comorbidities between compulsive drug seeking and anxiety/depression are well established [88,100,101]. I was, therefore, unable to determine whether pre-existing anxiety or anxiety in response to opioid withdrawal was more predictive of the development of an OUD.

In Chapter 4, I determine the anxiety state of each genetic strain of mice utilized in this study. I show that Arr3-KO and RMOR mice exhibit more anxiety pre-study, while only Arr3-KO mice exhibit increased anxiety post-study. I then show that like WT mice, anxiety state is not predictive of compulsive drug-seeking behavior in Arr3-KO mice. RMOR mice exhibited increased anxiety but not increased drug-seeking behavior. Taken together, this implies that modeling anxiety in rodents may be an inadequate representation of human mood disorders and their impact on the development of OUD. These data also warrant controlled follow-up experiments to determine if increased anxiety in Arr3-KO mice is reproducible in comparison to WT mice. Ranked social status in Arr3-KO mice, however, was predictive of compulsive drug-seeking behavior.

In Chapter 5, I show that morphine profoundly alters the gut microbiome. These changes are not reversible on the time scale of a month. I then identify several key bacterial species that were altered in response to morphine treatment and propose that they serve as biomarkers for compulsive drug-seeking. Identification of bacterial species that predispose an individual to an OUD has broad implications in reducing the number of persons who transition into having an OUD. Counteracting the specific changes described by the biomarkers of compulsive drug use with a targeted probiotic could reduce the number of people who transition into an OUD after being prescribed opioids.

II) Study limitations

This study has limitations. Many of the measures presented in this dissertation rely on the accurate calculation of a composite compulsivity score (Fig. 2.1E). The calculation of this composite score deviates from the standard practice for assessing drug-seeking behavior. Most studies monitor the total number of rewards earned or the total number of lever presses and follow behavior for less than one month [137–141]. The use of a jugular vein catheter to directly infuse animals with the rewarding drug immediately following a correct lever press is also commonplace. Animals while animals in this study drank morphine from a reward port. This could present a caveat, as the bioavailability of morphine is 23% of IV morphine and peak onset for oral drugs is about 45min [142]. Therefore, it could be said that lever pressing behavior in our study was more indicative of drug-pairing behavior than self-administration behavior. Most of the morphine self-administration in this study occurred in the home cage with no lever pressing required. Mice were cued in the operant self-administration boxes by the taste and other operant stimulus pairing but were not receiving therapeutic or rewarding effects while in the chamber, unlike mice with catheters.

The paradigm described in this dissertation deviated from typical for a few reasons: 1) A main goal was to monitor *long-term* drug use exhibited in humans with OUDs. Short-term drug use is more indicative of the binge/intoxication phase of addiction than a manifestation of the characteristics associated with the development of an OUD [143]. Jugal vein catheter patency over such a prolonged period would be immensely challenging. 2) Long-term access to morphine in the home cage allows mice to self-administer a great deal more than limiting access to operant sessions. 3) We had a goal of evaluating individual variations in behavior and what predispositions (like anxiety, social status, and microbiome) varies with drug-seeking behavior. Therefore, we wanted to include multiple measurements of drug-seeking behavior across a long duration to encapsulate chronic behavior. This prevented sole focus on one phase of

the paradigm, such as drug escalation, difficulty ceasing drug-seeking behavior, or increased relapse.

Another limitation is the use of whole-body transgenic mice as Arr3-KO and RMOR mice are both global knockouts. It is possible that the removal of Arr3 could cause other compensatory changes, such as the upregulation of other isoforms of arrestin, such as Arr2 [112,144]. Global knockouts could also cause dysregulation of other targets that are regulated by Arr3. For example, Arr3 also regulates dopamine D2 receptors which have been implicated in depression, drug addiction, and motivation [145–147]. Similarly, a major criticism of the RMOR mouse model has been that the extensive modification of the c-tail of WT MOR with the delta opioid receptor [67] could alter signal transduction. It is possible that this could result in modified signal transduction from the RMOR receptor that is not present in the WT receptor. For example, increased phosphorylation of the tail by PKA, recruitment of a different isoform of arrestin, or altered signal transduction affecting transcription could be mediated through the delta opioid receptor tail.

It can also be said that the inclusion of measures of physical tolerance and dependence on the effects of oral morphine would have provided good supporting evidence towards establishing compulsive in non-compulsive mice. As the definition of an OUD includes physical tolerance and dependence, the inclusion of these parameters would have strengthened the argument. A dose-response curve 24 hours after the last morphine dose, followed by injection of the MOR antagonist naloxone and assessment of physical withdrawal signs would have accomplished this.

III) Future directions for biased agonism and other theories

The search for safer opioids has yielded many competing theories. At the center of these theories is the controversy concerning the role of Arr3 in mediating the negative side effects of opioids. Although Arr3 has previously been postulated to mediate respiratory depression and reduce tolerance, these claims have not been reproducible [79]. My results indicate that Arr3-KO mice and WT mice exhibit identical levels of drug-seeing behavior solidify the idea that Arr3 does not mediate negative side effects. Our results recapitulate that increased Arr3 engagement is likely *beneficial* for the prevention of OUD correlates, like drug-seeking behavior [54]. Like the physiological response to endogenous ligands, the RMOR mouse effectively engages Arr3 after morphine binding, leading to the internalization of the receptor [27,53]. This likely prevents the desensitization of MOR on the membrane by properly recycling MOR and prevents the long-term cAMP increases that counteract continued G_i signaling. Prevention of cAMP super activation could prevent CREB activation and subsequent changes in gene expression that underlie neuroadaptations. This is supported by the fact that only RMOR shows decreased physical dependence [54,55].

However, mutation of the RMOR c-tail may signal to another binding partner. One way to test this hypothesis would be to perform experiments to test the development of tolerance and dependence and drug-seeking behavior in double-transgenic Arr3-KO/RMOR mice. If the reduction in negative side effects seen with RMOR mice is due to engagement with Arr3, then the double transgenic mice will not exhibit these same positive effects. If it is due to signaling to another effector, then the Arr3-KO/RMOR would develop these positive effects. These experiments are underway.

A more direct mutation of MOR would increase experimental evidence for the benefits of biased agonism. Conversion of T370 into a phosphomimetic, such as glutamic acid, would convert increase the likelihood of complete phosphorylation of the c-tail and subsequent Arr3 recruitment. Previous experiments suggest that morphine

induces phosphorylation of the first residue in the c-tail, S375, but not the second, T370. None of the studied ligands showed S375 and T370 to be phosphorylated without T376/T9 being phosphorylated [13,14]. Therefore, T370 might serve as a rate-limiting step in the complete phosphorylation of the c-tail. Conversion of T370E into a phosphomimetic will most likely result in complete phosphorylation and subsequent Arr3 recruitment, just like in RMOR but with less modification of the c-tail. *In vitro* assays validating this theory would be the first step. Commercially available phospho-specific antibodies to the c-tail phosphorylation barcode would be able to detect if the T370E mutation was able to produce a complete phosphorylation barcode in response to morphine. Then, assessment of cAMP super activation following chronic morphine treatment could provide a cellular analog for the development of physical dependence and tolerance. If the phosphorylation barcode was complete and there was an indication that the mutation was able to prevent cellular tolerance and dependence, follow-up studies in a T370E rodent model would be logical. Since the mechanisms leading to the development of many of the negative side effects are not fully resolved, a mouse model would allow us to assess these.

Another method to study the effect of biased agonism would be to evaluate the side effect profile of methadone in Arr3-KO mice. Methadone is commonly used as an adjunct therapy for OUD treatment due to lessened abuse liability [148]. It is unclear what properties of methadone confer this reduction in abuse liability, but it is known that methadone exhibits a signaling profile more closely related to enkephalin than morphine [51] and can recruit Arr3 [149]. Methadone-occupied MOR also exhibits a vastly different confirmation as compared to morphine-occupied MOR [83]. Taken together, this evidence suggests that there is something different about methadone that warrants additional studies. If the signaling profile evoked by methadone was balanced, similar to RMOR mice, I would hypothesize they would develop less analgesic tolerance and physical dependence than WT mice. If the reduced side effects seen in methadone

are mediated by Arr3, then I would predict that profound tolerance and dependence would return in Arr3-KO mice. Additionally, repeating the oral operant self-administration paradigm in this dissertation in Arr3-KO mice with methadone would determine if the reduced abuse liability of methadone was due to Arr3 recruitment.

Another important question is to answer how different cellular signaling events are in circuits controlling respiratory depression, analgesia, and reward. Tolerance to the respiratory suppressive effects of opioids develops more slowly than to the analgesic effects. It would be interesting to quantify the bias factor of various drugs, as well as expression levels of GRK and Arrestin-3 and G-proteins, in respiratory centers such as the preBötzinger complex. This prevents extensive limitations as the preBötzinger complex is small- about 1,000-3,000 neurons/side in the rodent brain [150]. A good starting place would be to perform single-cell RNA sequencing experiments in this complex of neurons and compare the changes in gene expression to changes in gene expression in the mesolimbic dopamine system. Identification of differential regulation or expression of genes in these two subtypes could lead to more targeted therapeutics that avoid activation of cellular signaling events in respiratory centers and reward centers while maintaining analgesic relief.

Of course, the development of a MOR ligand that mimics endogenous signaling, making it a balanced ligand, would be the most direct way to study biased agonism. This ligand would have to have a G-protein and Arr3 profile that mimics endorphin both in EC50 and Emax. Although the exact potency would not have to be the same, the relative activation of each would have to be. It would also need a wide enough safety profile to induce profound respiratory depression at analgesic doses. This is the case with fentanyl. Fentanyl can recruit Arr3 effectively but is limited by a small safety window and induces profound respiratory depression. In addition to all these criteria, this ligand would also have to be well tolerated by a general population and not have the variability in metabolism seen in methadone. This goal remains elusive, as difficulties

in assessing bias factor at multiple effectors remain and pharmacokinetic limitations are harder to predict pre-clinically. I propose the development of a ligand that encompasses all three major theories. This ligand would have the following characteristics: 1) prevention of MOR desensitization through the engagement of Arr3, 2) lower intrinsic efficacy than fentanyl to avoid respiratory depression, and 3) G-protein activation that mimics a balanced signaling profile.

IV) Alternative therapeutics

It is possible a hypothetical ligand able to maintain analgesia while reducing abuse liability, and not inducing severe respiratory depression is not achievable. The current treatment of OUDs involves prescribing opioids with less severe side effects, such as methadone or buprenorphine to people experiencing OUDs. This helps to prevent physical withdrawal and craving but does not offer total abstinence. Several other therapeutics that aim to mitigate other aspects of opioid use have been proposed such as I) prevention of neural adaptations that underlie opioid abuse disorder, II) vaccinations and long-lasting agonists that prevent further use, and III) probiotic supplementation to prevent systemic inflammation. For example, the prevention of neuronal adaptations by limiting changes induced during opioid use is a promising area of research. Epigenetic regulation of genes is altered during substance abuse through chromatin remodeling, methylation, and microRNA regulation [151]. Post-mortem assessment of human brain tissue revealed chromatin accessibility alterations resulting in the upregulation of a tyrosine kinase, FYN, in response to long-term heroin use. Treatment of rats with saracatinib, a FYN inhibitor, reduced the self-administration of heroin in rats [152].

Multiple vaccines that prevent opioids from exerting their therapeutic effects to combat repeated unnecessary medical use have been made. A hapten that is

structurally similar to the opioid ligand of interest is conjoined with a protein that produces an immune response. This effectively elicits an immune response to any opioids that enter the bloodstream and the opioid molecules are bound by the antibody, unable to cross the blood-brain barrier [153]. The use of long-acting antagonists such as methocinnamox (MCAM) offers a similar treatment option by blockading opioid receptors from being occupied to prevent reward and overdose [154,155]. Major limitations of these include specificity to opioids, which is problematic in polysubstance abusers, and voluntary continuation of treatment. These therapeutics do not prevent the development of an OUD or treat the underlying cause; however, they are effective at preventing overdose death.

As outlined in Chapter 5, probiotic supplementation could offer some protection against the development of OUDs. Supplementation of probiotic species shown to reduce compulsive drug-seeking behavior in our model could be given during opioid treatment. To do this, further quantification of the individual bacterial species identified from the samples produced from this study would need to be accomplished. Further analysis using whole genome sequencing will be used to detect species-level alterations and further identify biomarkers. The next step would also include using fecal material transplant of mice identified as non-compulsive into opioid-naive animals to determine if the proportion of compulsive drug-seeking mice can be reduced with modification of the microbiome. If these experiments are successful, the formulation of a probiotic pill taken in conjunction with opioid treatment could help reduce the ao

V) Final Remarks

The opioid epidemic continues to impact the lives of countless individuals suffering from opioid use disorder, as well as their loved ones and communities. The search for opioids with reduced abuse liability remains elusive, but I hope that the results presented in this dissertation progress the field incrementally. This dissertation

provided evidence that Arr3 does not exacerbate drug-seeking behavior, but rather improves it. The inclusion of Arr3 as a screening criterion in the development of future opioids should be paramount. In addition, further work defining the changes induced by morphine in the gut microbiome provides a meaningful avenue for mitigating inflammatory response and, potentially, reducing the development of OUD. Evaluation of anxiety state in a rodent model may prevent significant limitations in translating conclusions to human behavior, but the determination of the directionality of comorbidities and how to treat them effectively also has far-reaching implications for the well-being of those suffering from OUDs. A future where OUDs are preventable, or extremely treatable, is a distinct possibility.

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