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Revisiting opioid hypotheses and bridging contradictions with multifaceted experimental strategies and relevant laboratory models

By

SARAH WARREN GOODING
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

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This work is dedicated to the victims and survivors who have suffered under the war on drugs and the capitalist greed that birthed our American overdose crisis.

May you and all who love you find peace and dignity.

Abstract

In recent years, opioids have become a household name as America has faced down an unprecedented wave of overdose death. The fallout of the emergent public health crisis known as “the opioid epidemic” has a storied past stretching well into the last century. Despite a long history of human interest and scientific study of opioid drugs, the current state of biological opioid research has little to offer in service to reducing the scope of this ongoing tragedy. The intent of this work is to enhance the perspective of the fields of opioid pharmacology and neuroscience, both by encouraging routes of investigation which break from tradition, and casting sharper focus on studies with high relevance to real world scenarios. We present data from our own studies which employ novel strategies to examine behavioral and neural signaling changes that arise with acute and chronic opioid exposure. Additionally, we conduct extensive review and analysis of the domains of the opioid literature that inspired these studies, prioritizing the resolution of key controversies and illuminating scholarly gaps that necessitate further inquiry.

Acknowledgements

I can say with total confidence that this work could not have happened without the help and support of so many people. First on the list is Jennifer Whistler, without whom I cannot imagine the scientist I am today. From the first day in her lab I was encouraged to cut against the grain. The trust and confidence placed in me to boldly step beyond the purview of her research group lay down the dominoes I needed to discover my independece, learn when to ask for help, and let the data speak for itself. Perhaps her greatest achievement of this decade is putting together a team that truly cares for one another. Even at the times when I felt adrift on my own experimental island, the Whistler lab team was my steadfast mooring. There is nothing quite like working with people you enjoy and so much of the joy I found in my work is thanks to this irreplaceable crew.

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Table of Contents

Abstract ***iii***

Acknowledgements ***iv***

Chapter 1 Introduction ***1***

1.1 We are the pathogen; we are the cure: an epidemic of opioid overdose **1**

1.2 Angels of life and death: the poppies and thorns of opioid drugs **4**

1.3 Classifying opioid use: an incomplete toolbox **7**

1.4 References **9**

Chapter 2 A balancing act: Learning from the past to build a future-focused opioid strategy ***11***

2.1 Introduction: A centuries-long search for better analgesics **11**

2.2 Signaling and regulation of the mu-opioid receptor **15**

2.3 A biased view of opioids **21**

2.4 Exploiting endogenous mechanisms for improved outcomes: Does nature know best? **23**

2.5 Barriers to consensus: the specifics and semantics of pharmacological bias **29**

2.6 Moving forward with a reconciled view of contradictory results **34**

2.7 Opioids research must prioritize human outcomes **43**

2.8 Acknowledgements **44**

2.9 References **44**

Chapter 3 Deletion of arrestin-3 does not improve compulsive drug-seeking behavior in a longitudinal paradigm of oral morphine self-administration ***51***

3.1 Introduction **51**

3.2 Methods **54**

3.2.1 Mice 54
3.2.2 Determination of physical dependence to oral morphine 54
3.2.3 Generation of oral dose-response curve to morphine 54
3.2.4 Operant Training with saccharin reward 55
3.2.5 Oral Morphine Consumption Schedule 56
3.2.6 Operant Oral Self-Administration Weekly Schedule 56
3.2.7 Extinction 57
3.2.8 Abstinence and Reinstatement 58
3.2.9 Calculation of compulsivity composite scores 59
3.2.10 Morphine Preference 60
3.2.11 Statistical Analyses 60
3.2.12 Study Approval 60

3.3 Results **61**
3.3.1 Oral morphine self-administration is sufficient to produce both analgesia and dependence. 61
3.3.2 Deletion of arrestin-3 does not reduce drug-seeking behavior in a longitudinal OUD model. 62
3.3.3 Arrestin-3 deletion does not improve compulsivity as defined by a behavioral composite score. 67
3.3.4 Compulsive drug-seeking behavior is independent of morphine consumption or preference. 71

3.4 Discussion **73**
3.3.1 Arrestin-3-MOR activity does not cause or exacerbate compulsive drug-seeking 73
3.4.1 Opioid reward is an insufficient indicator of abuse liability 74
3.4.2 Balanced agonism is an under-studied strategy with potential to improve opioid therapeutics 75

3.5 Acknowledgements **78**

3.6 Author contributions **78**

3.7 Funding and competing interests **78**

3.8 References **78**

Chapter 4 Placeholder for photometry chapter *Error! Bookmark not defined.*

4.1 Introduction **81**
4.1.1 Opioids disinhibit the dopaminergic neurons of the ventral midbrain 81
4.1.2 Chronic opioid exposure alters opioid-sensitive GABA neurons 82
4.1.3 The mesolimbic circuit 84
4.1.4 Chronic opioids and dopamine release 85
4.1.5 Dopaminergic heterogeneity 90
4.1.6 Modernizing opioid pharmacology with neuroscience techniques 92

4.2 Methods **93**
4.2.1 Mice 93
4.2.2 Drug Preparation 94
4.2.3 Stereotaxic Surgeries 94
4.2.4 Jugular Vein Catheterization 95
4.2.5 Fiber Photometry 96
4.2.6 Photometry Analysis 96
4.2.7 Daily Injections 97
4.2.8 Histology 97

4.3 Results… **98**

4.4 Discussion **108**
4.4.1 Dopamine release is distinctly altered by morphine injection in the medial and lateral shell of the NAc 108
4.4.2 The mechanisms of opioid tolerance in the NAc still require extensive investigation 110
4.4.3 Unique challenges accompany the search for neural correlates of opioid withdrawal 112
4.4.4 Other considerations for future interrogation the relationships between opioids and dopamine release 114

4.5 Acknowledgements **116**

4.6 References **116**

Chapter 5 Conclusions, Caveats, and Future Directions ***121***

5.1 Conclusions at a Glance **121**

5.2 Re-examining opioid drug development with a balanced signaling profile **122**

5.3 Prioritizing disease-relevant behaviors in studies of signaling bias and side-effects 123
5.3.1 Caveats and Future Directions of Chapter 3 124
5.4 Applying optical neuroscience techniques to the pharmacological study of chronic opioid use 127
5.4.1 Caveats and Future Directions of Chapter 4 127
5.4.2 Challenges of using an optical method to study pharmacology 132
5.5 Closing Remarks 136
5.6 References 137

Chapter 1 Introduction

1.1 We are the pathogen, we are the cure: an epidemic of opioid overdose

The American opioid crisis is one of the defining tragedies of the last generation. In the last two decades, opioid overdose has devastated communities across America, causing massive healthcare burdens and claiming hundreds of thousands of lives (Fig. 1). According to the CDC, there were 80,411 opioid overdose deaths in 2021, or 220 deaths per day, accounting for more than 75% of all drug overdose deaths [2]. These deaths have a broad demographic reach, with high rates across disparate age and ethnic

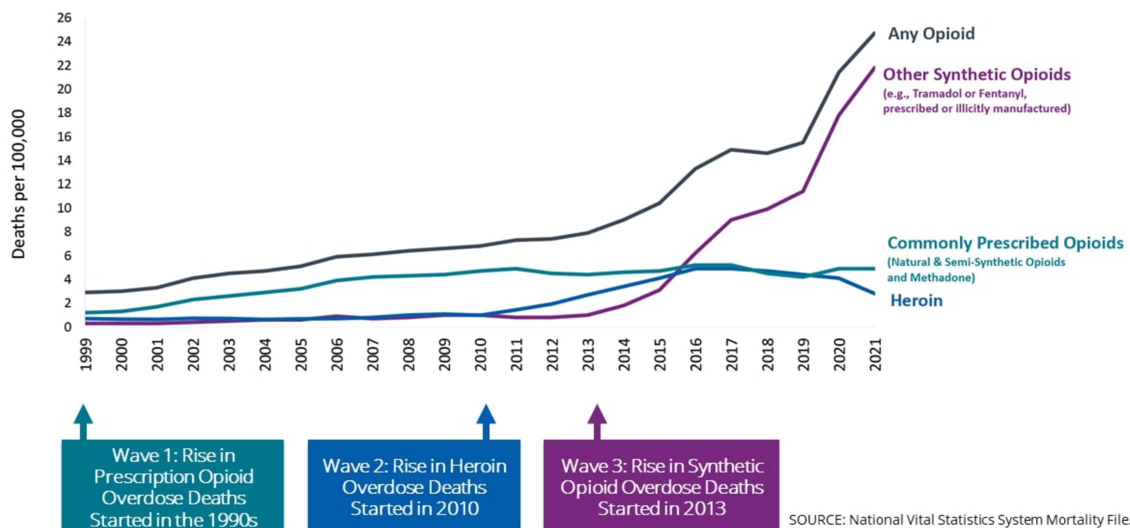

Figure 1: Annual US opioid overdose deaths per 100,000 people since 1999 [1].

groups [3]. For every fatal overdose, there are several non-fatal overdose events with their own wake of personal and economic harm. Beyond the acute consequences to people who use drugs and their loved ones, ripple effects such as the strain on first

responders [4, 5] cause downstream impacts on healthcare and criminal justice systems and through society at large.

The contemporary opioid crisis is comprised of three phases. The first, beginning in the late 1990s, was characterized by a steady rise in overdose deaths involving prescription opioids and lasted approximately a decade [6]. This rise corresponded with a massive increase in the number of opioid prescriptions issued by clinicians during this time [7]. These prescribing practices represented a good-faith effort by physicians to better prioritize the pain management of patients and accompanying recommendations from the medical establishment [8]. However, it has since been established that these medical practices were largely engineered and encouraged by profit-driven interests in the pharmaceutical industry. Following the FDA approval of Oxycontin in 1995, which allowed the drug to be labeled for indications beyond what were appropriate [9], Purdue Pharma launched a goliath marketing effort that hinged on fraudulent claims that the drug lacked the abuse liability of other opioid pain-killers [10]. The surge in opioid use resulted in a large population becoming dependent on these drugs, including both individuals who gained access through clinical use and those who obtained them through diversion of prescription drug supplies.

Around 2010, a second phase of overdose deaths emerged in which heroin was the primary lethal substance [11]. This rise in heroin overdose deaths is believed to be a consequence of the increased non-medical opioid use that naturally followed the aforementioned increase in availability of prescription opioid drugs [12] but is likely also related to a shift in the relative cost and availabilities of opioid pills and illicit heroin [13].

Unlike the heroin use of previous decades, individuals who began using heroin in the 2000s were likely to have first used prescription opioids [14].

Shortly after this uptick in heroin deaths, overdoses involving synthetic opioids such as fentanyl began to appear and within a few years became the dominant variety of fatal overdose [15]. Fentanyl is a highly potent opioid which can be lethal at doses 100 times smaller than the lethal dose of heroin. Fentanyl can be manufactured in a lab with relative ease and low cost compared to opiates such as heroin which must be extracted from a poppy. This makes it a popular add-in as a cost-cutting measure in the illicit drug trade where it has been increasingly detected throughout the last decade [16]. The pharmacological potency of fentanyl combined with its widespread, often unintentional use has driven an exponential increase in overdose death prompting the declaration of a United States public health emergency in 2017 [17].

The harm wrought by the opioid epidemic has been amplified by overlapping timing of its onset within an era of American policy deeply influenced by the campaign termed the “war on drugs”. Beginning in 1971 during the Nixon administration, federal drug policy has emphasized a carceral approach to combatting the production, distribution, and use of illegal drugs. These policies have prioritized criminalization over the expansion of medical and social resources to assist individuals suffering from drug dependence. The war on drugs has been characterized as a policy failure [18] and is widely criticized for shirking evidence-based practices in favor of legal and political projects that are unabashedly unjust, racist, and cruel. These movements have championed a cultural climate that, through both social stigma and fear of legal repercussion, pushes drug use

into the shadows. The criminalization and stigma serve as barriers to treatment and harm reduction resources and encourage individuals to use drugs in the most dangerous way possible: alone.

1.2 Angels of life and death: the poppies and thorns of opioid drugs

An opioid is any molecule that binds to and changes the activity of one or more of the opioid receptors. Opioid receptors are expressed throughout the brain, spinal cord, and peripheral nervous system where they serve modulatory roles in emotion, pain perception, respiration, and gut motility. There are several endogenous peptide opioids produced in neurons and integrated into the natural function of the body and brain. There is also a pharmacopeia of small molecule opioids, both naturally occurring and synthetic, that are frequently used both in clinical medicine and outside of clinical indication. Informal discussion of opioids almost always refers to small molecule drugs that are agonists of the mu-opioid receptor. These drugs are the primary topic of this dissertation and are hereafter what is meant by “opioids” unless otherwise indicated.

Opioids are known to promote a battery of behaviors and physiological side-effects that are colloquially referred to as addiction (clinically labeled substance use disorder [19]). Other substances commonly understood to promote this pathology are stimulants, amphetamines, hallucinogens, barbiturates, benzodiazepines, some cannabinoids, and alcohol. Opioids are medically essential but have a high propensity for acute lethality, and the thorny interaction of these features sets them apart from other substances of abuse.

For millennia, opioids have been used to alleviate human suffering. Opioids exert their analgesic effect by inhibiting nociceptive circuits in the spinal cord. This central blockade

of pain perception makes them highly effective in severe pain cases where non-opioid analgesics, such as anti-inflammatory drugs, are insufficient. To date, no other antinociceptive agents have been discovered with a comparable efficacy for treating severe pain. On a practical level, there are several water-soluble small molecule opioid drugs appropriate for compositions that allow for both hospital use and safe and effective oral medication. These factors combined make opioids an indispensable tool in clinical medicine. There is presently no alternative.

The acute danger of opioids comes from their undesirable impact on respiration. Opioid receptors are highly expressed in a brainstem nucleus called the pre-bötzinger complex which is responsible for autonomic control of breathing function [20]. Activation of opioid receptors has an inhibitory effect on neurons through mechanisms common to $G_{i/o}$ type G protein coupled receptors [21]. Because of the essential function of pre-bötzinger neurons in the generation of the breathing rhythm, their inhibition depresses the respiratory rate. At higher doses, opioids depress respiration to a point incompatible with the oxygenation of tissues needed to sustain life. This respiratory suppression is the mechanism of death in an opioid overdose. Given how little time the body can survive without oxygen, this leaves a very short window for lifesaving medical intervention.

All drugs are toxic at sufficient doses, a principle in toxicology known as “the dose makes the poison”. However, lethal opioid doses are significantly lower than other commonly used drugs like alcohol or cocaine [22]. This potential for the covert presence of high doses makes the inadvertent access and consumption of a lethal dose far more likely. The increased prevalence of synthetic opioids such as fentanyl in the drug supply

have amplified this factor as lethal doses have decreased by orders of magnitude from the previously favored morphine derivative drugs. As drug toxicity is also impacted by pharmacokinetic properties, use behavior practices such as route of administration also factors into the acute toxicity risk. Some opioids are frequently injected intravenously outside of medical settings, a practice less typical in the intentional consumption of other substances of abuse. Direct delivery to the bloodstream compounds the likelihood of overdose by maximizing the bioavailability of the drug and making its onset near-instantaneous.

Myriad social and cultural factors also bolster the likelihood of opioid overdose. As mentioned above, the stigma associated with non-clinical opioid use, especially intravenous drug use, makes individuals more likely to use opioids in isolation or in locations where help is less readily available. Use practices rooted in social shame or fear of law enforcement put people who use drugs at a far heightened risk of fatal overdose. Dependent individuals who obtain their opioids through prescriptions can be forced to switch to an illicit drug supply if they experience sudden loss of their prescription access. Such a shift is extremely hazardous because previous drug-taking practices and dose calibration can be incompatible with the doses present in opioids from unfamiliar sources. Relapsing drug use carries the same risks which are heightened by abstinence-based treatment programs that dissuade or disallow safer management strategies such as medically assisted treatment. Individuals who relapse are also more likely to use drugs in secret for fear of societal judgement or disappointing their support system.

1.3 Classifying opioid use: an incomplete toolbox

In the following chapters, opioid use is primarily discussed in the context of opioid use disorder (OUD) a diagnosis in the DSM-5 [19] designed to encompass the broad spectrum of pathologies that may accompany sustained opioid use. This diagnosis is discussed in more detail in chapter 2, but it is important to present the caveat that it does not sufficiently summarize the intricacies of opioid use in the population.

The DSM-5 is intended to create a standardized set of guidelines for psychiatric clinicians to use to categorize and direct patients into appropriate courses of treatment. This can be a useful framework in the medical field for continuity of care, and discrete diagnoses are often viewed as a necessary evil when working within the billing and insurance framework of the American medical system. However, it is not an infallible document. Its compilation is not beholden to heavy scrutiny by subject matter experts who prioritize the best available research, and its content is inevitably affected by the opinions, biases, and potentially even conflicts of interest of its authors.

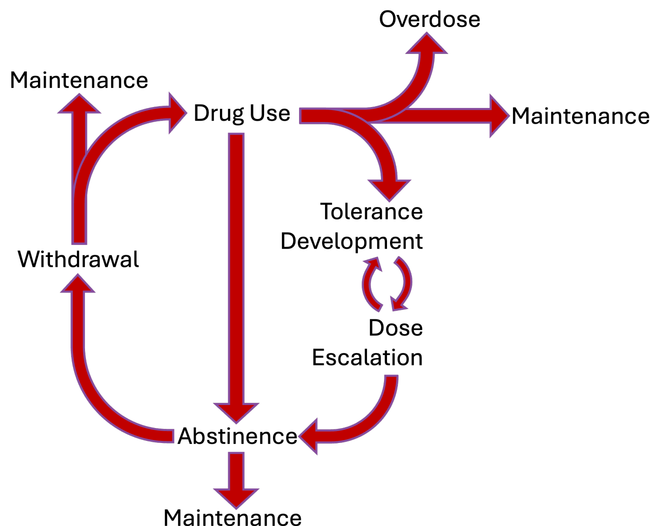

Figure 2: A holistic view of opioid use behavior patterns.

The institutional concept of OUD stands apart from conceptions of opioid addiction held by individuals or recognized by popular self-governed organizations such as Alcoholics Anonymous/Narcotics Anonymous. Such groups face

criticism for being unscientific in their methods and prioritizing tradition over adaptation, while the DSM-5 criteria may be seen as reductionist, rigid, and inaccessible to those without access to psychiatric treatment. No perfect system exists. Outside of clinical settings, opioid use may be therapeutic, recreational, compulsive, or any combination thereof and the trajectory of opioid use varies across the population (Fig. 2). When and whether it is problematic must ultimately be evaluated on an individual basis and such evaluation benefits from the perspectives of those with lived experience.

Viewing extraclinical opioid use through a disease framework can be helpful to humanize people who use drugs in the public perception by challenging the assumption that drug use is a moral failure. However, chronic illness is still widely stigmatized, and a different set of social challenges arises when drug users are indiscriminately labeled as "diseased". The harm reductionist framework is not concerned with litigating which drug-taking behaviors are problematic and instead seeks to mitigate harms that may arise after drugs are used. This is a useful angle because risks such as overdose can affect anyone who uses opioids, not only those who meet certain diagnostic criteria or have a chronic use history.

This dissertation discusses the actions of opioids at the molecular, neural circuit, and behavioral levels and presents a combination of our primary research data and perspectives synthesized from extensive review of the existing literature. The common thread herein is advocacy for improving models of opioid use to better serve the interests of affected individuals. This includes re-examining long-established hypotheses when they are called into question by new data. We also advocate for a range of models

(contingent/non-contingent, acute/longitudinal, behavioral/physiological) and mixing models when appropriate to better capture the diversity of opioid use behaviors and consequences. Finally, we encourage more targeted research with the intention of reconciling contradictory results throughout the opioid literature. In doing so, we aim to foster a more comprehensive understanding of opioid use that informs evidence-based interventions and policies, ultimately promoting the well-being of all those negatively impacted by opioids.

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Chapter 2 A balancing act: Learning from the past to build a future-focused opioid strategy

Sarah Warren Gooding and Jennifer L. Whistler

2.1 Introduction: A centuries-long search for better analgesics

Opioid drugs are the standard of care for treating severe pain, making them some of the most widely used and clinically significant drugs in medicine. Many individuals who suffer from an opioid use disorder (OUD), colloquially known as "addiction" and broadly defined as loss of control of drug seeking, were first exposed to opioid drugs in a clinical context. In this way, opioids differ from other drugs of abuse: their use is often medically indicated and necessary. The molecular and neuronal mechanisms underlying the transition from opioid use to the opioid misuse/abuse that define an OUD remain poorly understood, but these mechanisms lie at the heart of the opioid epidemic. In the United States alone, there are over 180 daily opioid overdose deaths, and the rate has been climbing since the 1990s and dramatically accelerated during the COVID-19 pandemic [1]. The scale of this tragedy has promoted widespread interest in mitigating the side effects of opioid drugs so that their therapeutic benefits may be applied with a decreased risk to patients. Better drugs that relieve pain with minimal harmful side effects are the ultimate aim of analgesic drug development. To this end, much attention and research has been directed toward identifying ways to amplify the beneficial effects while reducing the side effects of opioid medications. Unfortunately, these efforts have met with little success. As we find ourselves in an age of unprecedented potential for drug development and screening, it is important not to forget what we have already learned from this field's

lengthy history and to incorporate those lessons into new approaches. Our endogenous opioid peptides already provide excellent pain relief without treatment-limiting side effects, a premise we find encouraging.

Humans have used opioids for pain relief for thousands of years. The first known uses being opium and tinctures of opium such as laudanum. It was in 1804 that the first active analgesic ingredient in opium, morphine, was isolated by Friedrich Sertürner [2]. It was already recognized at that time that repeated use of opium resulted in addiction. Although Sertürner originally theorized that the need for smaller quantities of purified morphine compared to opium would reduce the addiction risk, this was not the case, and he documented his own addiction to morphine. The semi-synthetic derivative of morphine, heroin (diacetylmorphine), was first synthesized in 1874 [3] and marketed by Bayer in 1898 as a “less addictive” opioid. This launched over 100 years of false claims and false hopes for a painkiller with low abuse risk (Fig. 1).

Additional early efforts to create less addictive opioids focused on various ways of derivatizing the natural opium extracts morphine and thebaine, creating several drugs we still use today. These include oxymorphone (1914, introduced as Opana in the US in 1955), oxycodone (1916, first introduced in the US in 1928 as part of Scophedal, then mixed with acetaminophen as Percocet, and more recently in a slow release formula as OxyContin), hydrocodone (1920, approved in the US in 1943 as Dicodid, and mixed with acetaminophen as Vicodin), and hydromorphone (1923, marketed as Dilaudid in the US from 1927) [4]. Because these semi-synthetic derivatives had abuse liability and still relied on precursors purified from a poppy, the next pharmaceutical quest was to identify fully

synthetic opioids. The first synthetic opioid, pethidine/meperidine (sold as Demerol), with a structure unrelated to the morphine/thebaine opioids, was patented in 1937 and approved for use in 1943. However its toxic metabolite counter-indicates its use from long term pain [4]. Simultaneously, during a shortage of natural painkillers in WWII, methadone was synthesized to reduce demand for opium and morphine [5]. Methadone's half-life is much longer than the natural product opioids, but its half-life is also highly variable in the human population [6]. This variability has limited its use as a first line analgesic, but it is

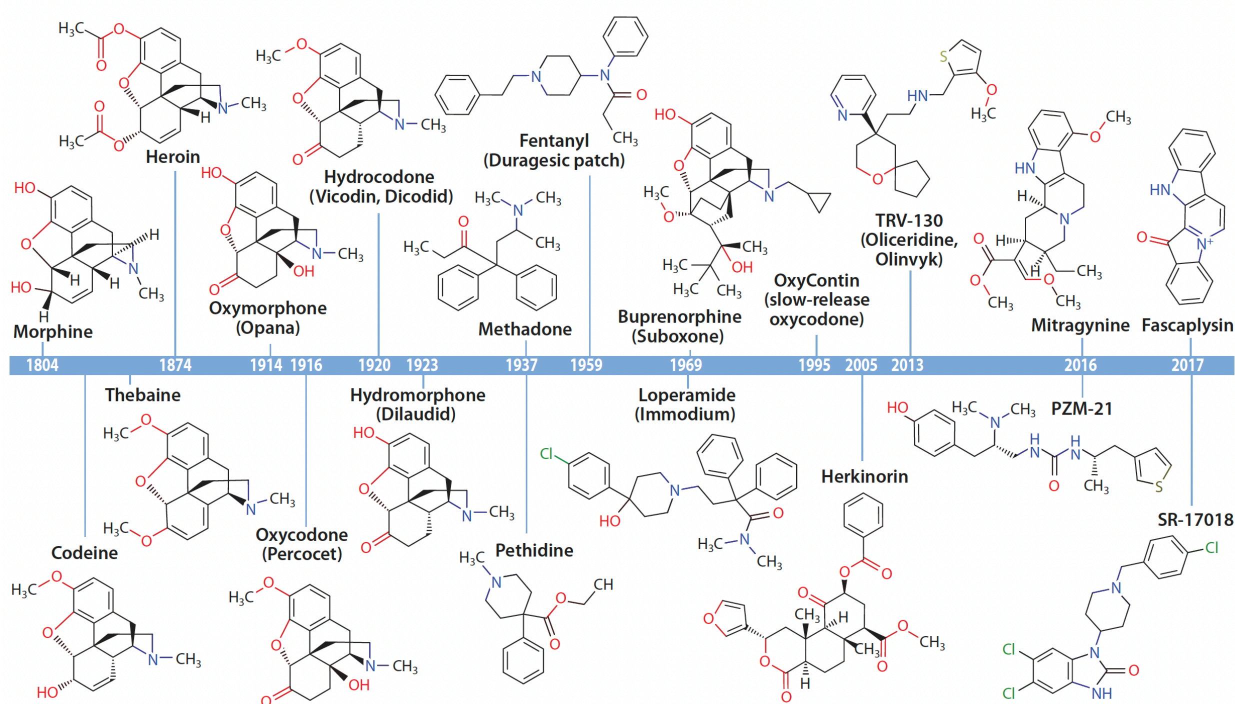

Figure 1: Historical timeline of opioid molecule discovery and synthesis.

still in widespread use for the treatment of OUD.

Two decades later, very high potency opioids were created, once again with the hypothesis that lower doses for pain would cause less addiction (as was initially assumed about heroin vs. morphine). This effort led to the synthesis of the fully synthetic opioid fentanyl and its derivatives (1959 and FDA approved in 1968) [7]. Contemporary efforts

also aimed to develop opioids with only partial agonism or mixed agonism/antagonism for the subtypes of opioid receptor. The hope was that these partial agonists might be less rewarding and therefore less addictive. These efforts produced the synthetic drug loperamide [8] (1969, with FDA-approval in 1976 as Immodium, an important anti-diarrheal due to its activity in the gut [9]). Loperamide is not addictive, but it is also non-analgesic because it is rapidly transported out of the central nervous system (CNS) by p-glycoprotein. Another semi-synthetic opioid, buprenorphine (1969), was never FDA-approved for pain treatment due to its poor analgesic ability compared to the other opioids, but was approved as Suboxone and Subutex for treatment of OUD in 2002 [10].

With these successes at creating new opioids but little progress separating the analgesic effects from the addictive effects, the search for new opioid molecules subsided. The next significant opioid launch was simply a reformulation of oxycodone as a slow-release oral drug, which was FDA-approved in 1995 without additional long-term tests. Purdue pharmaceuticals famously hailed OxyContin as non-addictive, purporting that its slow release wouldn't give a high. This false assumption is now credited for the inception of our current opioid crisis when clinical practices shifted to a strictly "pain-averse" model, and unrestrained prescription access created a new generation of patients dependent on opioids.

This hiatus in opioid drug development and prelude to the American opioid crisis was a highly productive time for basic research on opioids. This period saw the identification of the endogenous opioid peptides (1975-1977) [11-14] and the cloning of the four opioid receptors (1992-1994) [15-24] all of which are G protein-coupled receptors (GPCRs) of

the $G_{i/o/z}$ class. These discoveries enabled clarification of the precise mechanism of action of the existing opioid drugs. An important finding was that the analgesic effects of these drugs were mediated primarily by the mu-opioid receptor (MOR) [25] and that all the drugs described above are agonists at the MOR. With a known GPCR target, a pharmacopeia of opioids, and a plethora of experimental approaches, the field was poised to return to the search for safer pain relief.

2.2 Signaling and regulation of the mu-opioid receptor

The analgesic action of opioids is dependent on MOR activation of trimeric G proteins. As a $G_{i/o}$-coupled GPCR, the agonist-bound MOR promotes exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on G protein. The GTP-bound activated G protein inhibits adenylyl cyclase via the alpha subunit which in turn decreases levels of cyclic adenosine monophosphate (cAMP) and the activity of protein kinase A (PKA). This receptor also inhibits neuronal activity through activation of GPCR inwardly rectifying potassium (GIRK) channels, and inhibition of voltage gated calcium channels (Ca_v). Opioids can thus hyperpolarize neurons through GIRKs and prevent transmitter release by reducing calcium influx through Ca_v inhibition. At the same time, they control levels of second messengers. These effects are all mediated by G protein. The adenylyl cyclase, GIRK and Ca_v effects of opioids have been measured in many cell types in the CNS as well as in the gut and immune cells [26].

The strength and duration of this G protein signal is regulated by innate ligand properties such as off-rate and intrinsic efficacy but also by rate of GTP hydrolysis, which can be increased through activity by regulator of G protein signaling (RGS) proteins [27].

In addition, G protein signaling through the MOR, like that from most GPCRs, is regulated by a cascade of events that include direct phosphorylation of the MOR by GPCR kinases (GRKs) in response to ligand binding. Phosphorylation then facilitates the binding of arrestins. Arrestins, first discovered in 1986 as regulators of rhodopsin [28] and later of the beta-2-adrenergic GPCR [29], regulate signaling of most GPCRs. Arrestin recruitment to the receptor uncouples MOR from G protein and scaffolds signal transduction by other second messengers including extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs). In addition, arrestin attracts a protein scaffold for internalization/endocytosis of the receptor. MORs that have been internalized by endocytosis are then de-phosphorylated, de-liganded and recycled back to the cell surface for future activation [30].

Arrestin recruitment and desensitization and/or downregulation of MORs have received much interest as possible mechanisms of analgesic tolerance, which often results from repeated use of opioids. Because tolerance can necessitate dose escalation, increasing the risk for respiratory side effects and OUD, there was significant motivation to identify its underlying mechanisms. This has inspired many papers reporting changes in receptor quantity or measuring desensitization of receptor signaling during or following morphine treatment—both before and after arrestins were discovered and the MOR was cloned and could be expressed heterologously to isolate MOR-specific effects.

Distinct GPCR ligands can be differentially potent and/or efficacious at activating the G protein versus the arrestin signaling pathways (Fig. 3). This functional selectivity for one GPCR effector versus another was first described by Roth and colleagues in 1987

[31] and has since been demonstrated for many classes of GPCR and coined "signaling bias" to reflect a gradient rather than a binary. The phenomenon of selective efficacy for one effector versus another received much skepticism until some second-generation antipsychotics were shown to promote serotonin receptor (5-HT_{2A}) endocytosis despite being antagonists for G protein signaling [32]. Bias became relevant to opioids when in 1996 it was found that although the endogenous peptide agonists—and [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) a hydrolysis-resistant enkephalin used as a surrogate for the endogenous ligand—promoted MOR endocytosis, morphine did not [33, 34]. A series of subsequent studies demonstrated that this poor MOR endocytosis in response to morphine was due to low levels of GRK phosphorylation and arrestin recruitment at the MOR [35-37].

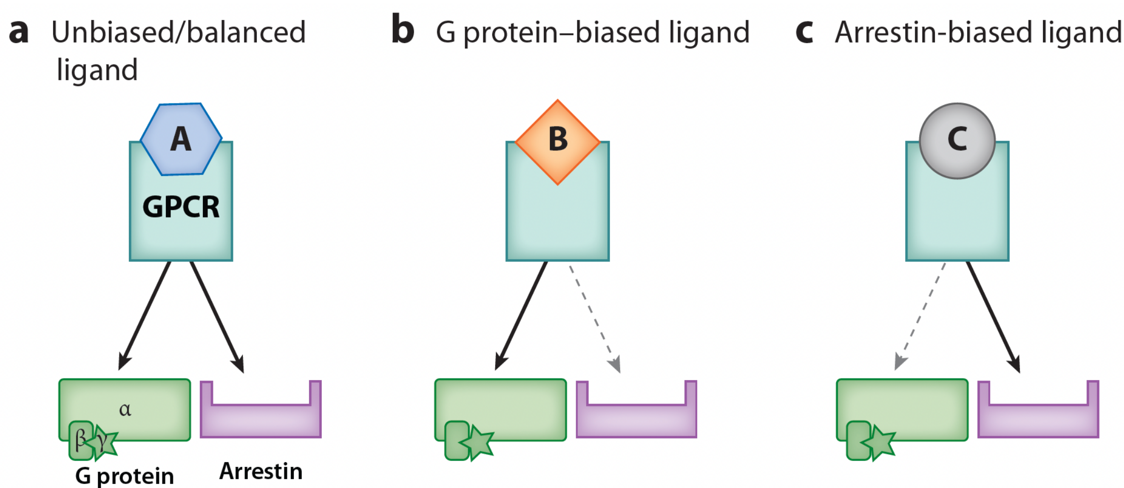

Figure 2: Schematic of balanced and biased agonism at an unspecified G protein–coupled receptor (GPCR). (a) An unbiased or balanced ligand (A) will cause the receptor to signal to both the G protein and arrestin pathways. (b) A ligand that is biased for G protein (B) will more effectively signal to the G protein pathway than to the arrestin pathway. (c) A ligand that is biased for arrestin (C) will more effectively signal to the arrestin pathway than to the G protein pathway. The arrow thickness indicates relative efficacy of signal compared to the other effector.

The discovery that opioid drugs, but not opioid peptides, display signaling bias for G protein suggested a pathway and possible mechanism to separate the beneficial from the

detrimental effects of opioids. Two competing hypotheses emerged from these results: that arrestins prevented the side effects and that arrestins were responsible for the side

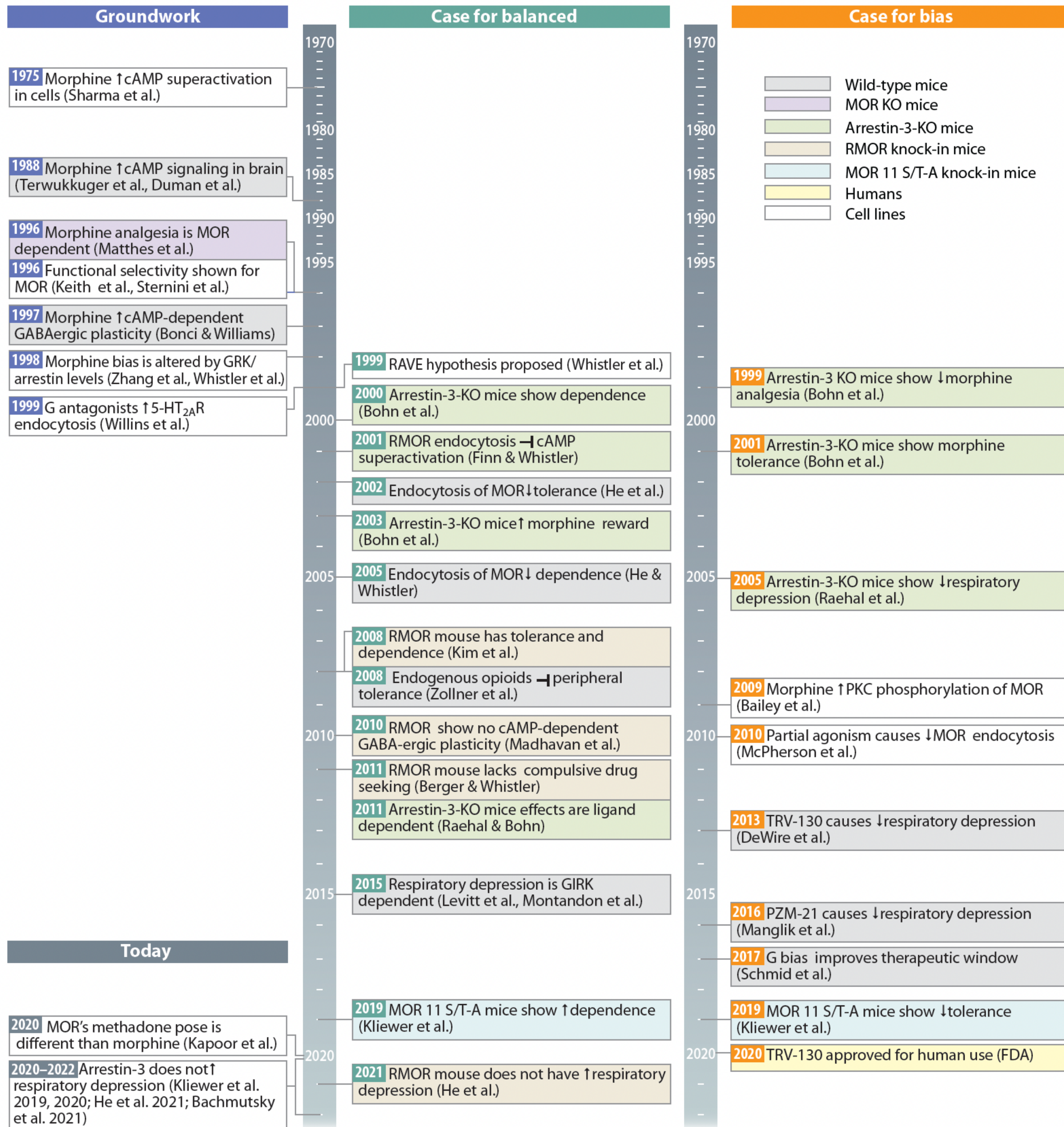

Figure 3: Historical timeline of key findings in support of balanced (green) versus biased (orange) opioid agonists. Hypotheses on the role of arrestin-3 diverged in 1999, and the field is now poised to begin a new era. Abbreviations: 5-HT2AR, serotonin 2A receptor; cAMP, cyclic adenosine monophosphate; GABA, gamma-aminobutyric acid; GRK, G protein–coupled receptor kinase; KO, knockout; MOR, mu-opioid receptor; PKC, protein kinase C; RAVE, relative activity versus endocytosis; RMOR, recycling MOR; TRV-130, oliceridine.

effects—a dichotomy that persists today (Fig. 3). The role of signaling bias in the effect/side-effect profiles of opioid drugs has been a focus of opioid drug development for more than two decades with significant resources directed towards the dominant hypothesis that arrestin-3 activity is responsible for the negative side effects of opioid drugs. While this view has fueled the development of several new ultra-G-biased opioid compounds, its premise has been challenged. Some groups maintain the position that balanced agonists have more therapeutic potential. Others attribute effect/side effect profiles to drug properties other than bias.

While the abuse potential of opioids is a side effect that garners much public attention, there are clear and meaningful limitations of discussing "addiction" in a basic research context. Substance use disorders, such as OUD, are complex human syndromes that present heterogeneously in the affected population. The Diagnostic and Statitstical Manual of Mental Disorders (DSM-5), the current authority for psychiatric disease, defines OUD as two or more of eleven diagnostic criteria presenting within a twelve-month period (Fig. 4). These criteria attempt to capture the range of the addiction experience and OUD may be classified, based on how many criteria are met, as either Mild (2-3 symptoms), Moderate (4-5 symptoms), or Severe (6 or more symptoms) [38]. Several of the DSM-5 criteria rely on a degree of self-evaluation and/or must be evaluated within a human cultural context which makes them difficult or impossible to evaluate in any model organism. Our discussion largely concerns the only two criteria on the list with direct physiological readouts: tolerance and withdrawal (dependence). We elaborate below on why mitigating these factors is particularly important when considering drug development

Diagnostic criteria	Animal model translation
1. Opioids are often taken in larger amounts or over a longer period than was intended.	No
2. There is a persistent desire or unsuccessful efforts to cut down or control opioid use.	No
3. A great deal of time is spent in activities necessary to obtain the opioid, use the opioid, or recover from its effects.	Yes
4. A craving or strong desire or urge to use opioids.	Yes
5. Recurrent opioid use resulting in the failure to fulfill major role obligations at work, school, or home.	?
6. Continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids.	?
7. Important social, occupational, or recreational activities are given up or reduced because of opioid use.	?
8. Recurrent opioid use in situations in which it is physically hazardous.	Yes
9. Continued opioid use despite knowledge of having a persistent or recurrent physical or psychological problem that it is likely to have been caused or exacerbated by the substance.	No
10. Tolerance, as defined by either of the following: a. A need for markedly increased amounts of opioids to achieve intoxication or desired effect. b. A markedly diminished effect with continued use of the same amount of an opioid.	Yes
11. Withdrawal, as manifested by either of the following: a. The characteristic opioid withdrawal. b. Opioids (or a closely related substance) are taken to relieve or avoid withdrawal symptoms.	Yes

Physiological | Psychological | Social/cultural

Figure 4: Diagnostic criteria for opioid use disorders (OUDs) as described in the Diagnostic and Statistical Manual of Mental Disorders: DSM-5™. The term addiction is used colloquially to refer to OUD. Here, we classify each diagnostic criterion as belonging to one of three experiential categories and by our ability to observe or model it outside of humans. Symptoms that have physiological readouts (10, 11) have well-understood models. Some psychological symptoms (3, 4, 8) can be modeled by various drug seeking or self-administration paradigms. Symptoms that require self-evaluation or communication of intent by a patient (1, 2, 9) do not have available models. It is disputable whether the social or cultural components of OUD (5–7) can be reasonably modeled outside of humans.

endpoints. However, much work remains to connect the cellular processes described herein with their cognitive and behavioral correlates to unveil a more complete understanding of addiction biology.

In this review we discuss the disputed relationship between arrestin-3 activity and opioid side effects and consider the complexities of pharmacology beyond bias. Furthermore, we attempt to reconcile disparate claims about the role of signaling and signaling bias in drug effect profiles and therapeutic window in a way that takes all the available data into account. It is our hope that the reconciliation of these claims might inform future directions of research and drug development.

2.3 A biased view of opioids

A key moment in opioid signaling bias research came with the observation that arrestin-3 knockout mice responded differently to morphine than their wild type counterparts with potentiated analgesia and reduced tolerance, among other effects [39-41]. This led to the hypothesis that receptor desensitization, which is regularly portrayed as the main source of tolerance in response to drugs, is mediated by phosphorylation and arrestin-3 recruitment to the MOR. Shortly thereafter, the same group demonstrated that morphine-induced respiratory suppression was attenuated by germline knockout of arrestin-3 [42]. This observation became the bedrock for a drug discovery strategy that prioritized the design of ultra-G-biased agonists to the MOR that promoted no arrestin-3 recruitment with the goal of mimicking the result of its genetic elimination. This occurred without independent replication of the result or further understanding of the mechanism behind the altered respiratory depression. In the last two decades, significant resources have been put behind this cause leading to the development of a few novel ultra-G biased agonists. One, oliceridine (TRV-130), has recently received approval for clinical use [43]. The primary goal of this research was to circumvent opioid-induced respiratory depression (OIRD), the cause of opioid overdose deaths.

Despite the early studies of arrestin-3 knockout mice that inspired a generation of research seeking to eliminate arrestin-3 activity at the MOR, the original respiratory results have proven difficult to replicate. A consortium of three independent laboratories across the world has reported intact morphine-induced respiratory depression in these mice [44], consistent with what our laboratory [45] and another [46] have observed. We sought to elucidate the mechanism that explains these differences and have postulated

that the mixed genetic background of the original arrestin-3 knockout mice is likely a source of their resistance to OIRD [45]. Improving upon the resolution offered by a germline knockout of arrestin-3, which undoubtedly alters signaling from receptors other than MOR, one group has also found no resistance to OIRD in a knock-in mouse that is incapable of recruiting arrestin-3 to the MOR due to substitution of all residues at key phosphorylation sites in the c-tail (MOR 11S/T-A) [47].

Additionally, our lab found that a panel of clinically relevant opioid analgesics with varying degrees of signaling bias all promoted respiratory depression at equi-analgesic doses in wild type animals with observable differences in the timing, but not the severity, of this effect [45]. A notable exception was buprenorphine, which produced little respiratory depression at equi-analgesic doses. Although buprenorphine is not used clinically as an analgesic, it is worth exploring whether this result represents a buprenorphine-specific signaling mechanism from the MOR, or its activity at targets other than the MOR (such as antagonism of the kappa-opioid receptor or activity at the nociceptin receptor). When imagining new opioid ligands, it is intriguing to consider that agonism at the nociceptin receptor can attenuate the rewarding effects of opioids [48]. This body of evidence presents a compelling case against arrestin-3 engagement at the MOR as the cause of OIRD. There is, however, recent work suggesting that morphine may cause additional respiratory depression in comparison to DAMGO [49], highlighting the need for more comprehensive respiratory studies exploring how endogenous and exogenous opioids contribute to OIRD in order to probe any role of bias that the aforementioned studies have missed. In the meantime, we believe that ample evidence

contradicting the foundational result upon which contemporary drug development strategies were based is grounds to reevaluate these strategies and broaden the search for mechanisms of interest.

2.4 Exploiting endogenous mechanisms for improved outcomes: Does nature know best?

While much of the field championed the pursuit of ultra-biased agonists, our group has pursued an opposing story: how enhanced arrestin-3 recruitment to the MOR alters signaling and *in vivo* responses to opioids (Fig. 2 upper timeline). Inspired by the observation that the endogenous ligands all engage arrestins but don't produce tolerance under conditions when exogenous drugs do, in 1999 we proposed the "RAVE hypothesis": that **R**elative **A**ctivity (at G protein) **V**ersus amount of **E**ndocytosis (in effect, signaling bias) would be predictive of tolerance and dependence to opioids. In this classification, endogenous peptide ligands have a low "RAVE" because G protein signal is titrated by arrestin/endocytosis, while the opioid drugs have a high RAVE because G protein signal is not opposed by arrestins. Our hypothesis was that ligands with a high RAVE would cause homeostatic adaptations to oppose MOR signaling and that these would manifest as tolerance in the presence of drug and dependence upon withdrawal of drug (Fig. 5). Simply put, chronic signaling through G_i, un-titrated by arrestin-3, would demand a rebalancing to homeostasis. One such homeostatic adaptation is cAMP superactivation, a compensatory increase in cAMP levels following prolonged adenylyl cyclase inhibition, a well-established cellular hallmark of morphine tolerance [50-54].

Shortly after we proposed high RAVE would produce tolerance (and dependence), arrestin-3 knockout mice were shown to have enhanced analgesia and reduced analgesic

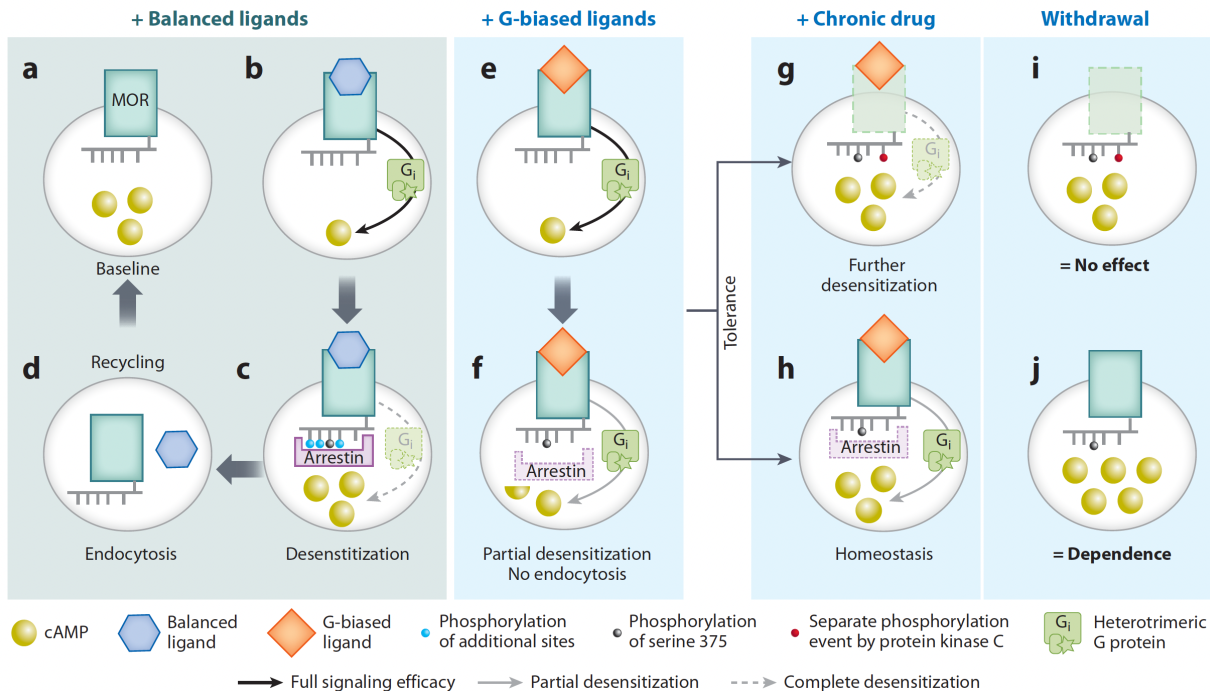

Figure 5: Schematic of opioid tolerance produced via desensitization versus cellular homeostasis. (a–d) Signaling response of MOR to balanced ligand (blue hexagons). (a) Empty receptor and baseline levels of cAMP (yellow spheres). (b) Balanced agonist at MOR promotes signaling to Gi, decreasing cAMP compared to panel a. (c) Complete phosphorylation of MOR (gray and light blue spheres) and strong arrestin recruitment lead to rapid desensitization of the G protein signal and return to baseline cAMP levels. (d) Endocytosis and recycling of MOR lead to rapid resensitization. (e–j) Signaling response of MOR to biased ligand (e.g., morphine; orange diamonds). (e) Acute morphine promotes signaling to Gi, decreasing cAMP compared to an empty receptor (panel a). (f) Single phosphorylation of MOR on serine 375 (gray spheres) and poor arrestin recruitment lead to weak desensitization of G protein. Once receptors are phosphorylated and partially desensitized, they remain this way without endocytosis and resensitization. (g,h) Chronic morphine produces tolerance by two mechanistically distinct processes. (g) Tolerance via further receptor desensitization. PKC phosphorylates MOR (red spheres), further uncoupling it from G protein and leading to higher cAMP levels (yellow spheres) in the presence of morphine compared to acute morphine shown in panels e and f. (h) Tolerance via homeostasis. Cells increase cAMP levels by mechanisms independent of MOR signal (e.g., increased adenylyl cyclase, decreased cAMP phosphodiesterase, increased signaling via Gs-coupled receptors). This also leads to higher cAMP levels (yellow spheres) in the presence of morphine compared to acute morphine shown in panels e and f. Tolerance via further desensitization (g) and tolerance via a homeostatic shift (h) are therefore indistinguishable in the presence of morphine. (i,j)Withdrawal following tolerance by the two mechanisms shown in panels g and h. (i)Withdrawal of morphine has no effect on cAMP levels when tolerance is produced only by receptor desensitization because the receptors are not functional. cAMP levels are the same as baseline seen in panel a. (j)Withdrawal of morphine causes an increase in cAMP (yellow spheres) to superactivation levels above those at baseline in panel a, revealing both the presence of the homeostatic shift and that MORs are still functional and controlling levels of cAMP. This cAMP superactivation manifests as withdrawal signs of dependence. Abbreviations: cAMP, cyclic adenosine monophosphate; MOR, mu-opioid receptor; PKC, protein kinase C.

tolerance to morphine, emboldening efforts to demonstrate that desensitization of MORs alone creates tolerance. Importantly, these are not mutually exclusive mechanisms (Fig.

5) and could occur simultaneously, either in the same cells, or possibly separated by cell type.

To understand the relationship between trafficking of the MOR and the effects of opioids, we co-administered DAMGO with morphine in wild type rats and found it prevented analgesic tolerance [55]. We hypothesized that this was due to homo-dimerization of the MOR wherein one receptor occupied by DAMGO is sufficient to recruit arrestins to the morphine-occupied receptors. The MOR has since been shown to dimerize in a ligand-dependent manner [56]. We expanded these studies using methadone [57], the only FDA-approved opioid analgesic that approaches the balanced signaling of the endogenous peptides [58], in hopes of creating an achievable therapeutic strategy. Rats given a cocktail of morphine spiked with methadone at doses that provided no additional analgesia do not develop tolerance or dependence, an effect we showed was independent of methadone's activity at the N-methyl-D-aspartate (NMDA) receptor [57]. Histology demonstrated that this dual opioid cocktail promoted endocytosis of the MOR while neither morphine, nor the sub-analgesic dose of methadone used in the study, were sufficient to do this on their own [57]. These results complement those suggesting that some pain patients are protected from tolerance and dependence to exogenous opioids due to naturally elevated levels of endorphins and enkephalins. This has been shown in rodent models of tolerance during inflammatory pain [59].

Given the lack of drug-like ligands with which to further interrogate the downstream effects of balanced opioid signaling, we turned to a genetic approach with the development of RMOR (for **R**ecycling mu-opioid receptor). RMOR is a chimeric receptor

containing a 22 amino acid substitution in the cytoplasmic tail with a sequence from the closely related delta-opioid receptor (DOR). This substitution gives enhanced arrestin-3 binding capacity while the G protein signaling is unchanged. In effect, signaling and trafficking of the RMOR responds to morphine much like the wild type MOR responds to DAMGO [60]. While creation of this receptor was done stochastically, we now know this sequence replaced the phosphorylation bar code of the MOR [61] 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 [35]. The phosphorylation barcode for robust arrestin-3 recruitment was carefully interrogated in 2018 [61]. Briefly, this group demonstrated that the wild type MOR is phosphorylated on 4 distinct residues in response to DAMGO while morphine-occupied MORs are phosphorylated on only one of these (S375), unless GRKs are highly overexpressed.

With this RMOR tool in hand, we could pursue our hypothesis without the caveats inherent to comparing ligands that differ in other pharmacological properties beyond bias. In cell-based assays, we demonstrated that cells expressing the MOR but not RMOR show cAMP superactivation, a key component of tolerance and dependence [60]. We then created a knock-in mouse expressing the RMOR. In 2008, we reported that RMOR mice were highly resistant to morphine tolerance after repeated dosing and did not exhibit withdrawal behaviors precipitated by naloxone [62]. We then demonstrated in the RMOR mouse model that adaptations following chronic morphine are prevented when signaling is altered in the direction of the endogenous ligands [63]. This includes cAMP superactivation, which is necessary for withdrawal behaviors [64-67]. We had previously

shown that methadone and DAMGO promote reduced cAMP superactivation compared to morphine [55, 60]. This supported our hypothesis that balanced signaling and MOR recycling impedes the cellular conditions that lead to tolerance and dependence. In slice electrophysiology studies, we found that the ventral tegmental area (VTA) dopamine neurons of RMOR mice were not subject to potentiated inhibition [64], a form of homeostatic plasticity that appears during opioid withdrawal [68-70]. Mice lacking arrestin-3 also show potentiated inhibition, even when opioid drug naïve, suggesting that endogenous ligands can cause similar plasticity as morphine when MORs are unable to engage arrestins [71]. Finally, despite potentiated analgesia and reward in response to morphine, RMOR mice do not transition to a pattern of compulsive drug taking behavior in a complex operant administration model of OUD [72], indicating a promising connection between receptor trafficking and abuse liability.

The pharmacokinetics of morphine—as well as any off-target effects—are unaltered in RMOR mice, and RMOR and wild type mice have the same number of opioid receptors [62]. The efficacies of morphine and DAMGO are equivalent both for activation of GIRK channels and inhibition of transmitter release in the VTA [64]. Furthermore, the 22 amino acid substitution is entirely contained within exon 3, meaning any putative MOR splice variants [73] that alter their endocytosis [74] also carry this new GRK barcode. While we cannot rule out that the RMOR, but not the wild type MOR, signals to an unidentified effector specific to DORs to protect against tolerance, dependence, and compulsive drug seeking, this seems unlikely as deletion of the DOR actually reduces tolerance to morphine [75]. Also, while morphine analgesia [62] and reward [72] are enhanced in

RMOR mice compared to wild type, methadone analgesia and reward are indistinguishable. All these data indicate that the change in signaling bias with morphine in RMORs, rather than a change in general RMOR signaling, is responsible for the reduced tolerance, dependence, and compulsive drug seeking in RMOR mice.

These findings arose during a period of opioid history when elimination of arrestin-3 activity in order to reduce side effects was the dominant hypothesis and drug development strategy (Fig. 2). As mentioned above, the goal at the time was to ameliorate OIRD with an ultra-G-biased signaling profile. If indeed OIRD was a direct result of engaging the arrestin-3 pathway, one would expect RMOR mice, with their enhanced arrestin-3 recruitment, to have exacerbated respiratory suppression on opioid drugs. However when we tested this, we found that OIRD was slightly exacerbated in arrestin-3 knockout mice compared to wild type mice [45]. RMOR mice had a respiratory response indistinguishable from wild type mice, strengthening the hypothesis that arrestin-3 activity is not causal for OIRD. As the previously favored hypothesis that arrestin–3 engagement produces respiratory depression has now been broadly overturned, and OIRD is widely believed to result from G protein activity [76, 77] (inseparable from analgesia), now is an ideal time to reexamine the implications of the RMOR results. From our perspective, this means prioritizing signaling that is balanced in accordance with the endogenous ligands as a possible avenue for reducing critical side effects including tolerance, dependence, and abuse liability. This would be unprecedented, and as mentioned previously, methadone is the only clinically-utilized opioid with a signaling profile similar to the endogenous ligands, though it is rarely used as a first line analgesic. However, in the few

human studies where methadone and morphine were compared in opioid naïve pain patients (patients who had not undergone morphine-induced changes in plasticity causing tolerance and/or dependence), methadone showed less tolerance and less severe withdrawal [78].

2.5 Barriers to consensus: the specifics and semantics of pharmacological bias

The stakes of this topic are professionally, financially, and ethically high, so it is unsurprising that the climate around this work, and the discussion of bias in particular, has grown contentious as contradictory results come to light. Thoughtful research is needed to address the apparent incompatibility of results across laboratories in order to move toward a common understanding.

At the core of this controversy is the debate over whether bias for G protein is or is not the explanation for side effect reduction, as some studies claim [42, 43, 79, 80] and others refute [44, 45, 47, 81]. The present trend is to explain conflicting results through differences in how bias is quantified [82-84]. One competing hypothesis that has gained recent momentum is that side effects are driven by the intrinsic efficacy of opioid agonists [83] and that the correlation of G protein activity and arrestin-3 recruitment [85] has led to the misattribution of these effects to arrestin-3 activity. While this may be true, some supporting experiments involved the overexpression of GRKs which alters the efficacy of arrestin-3 recruitment and, by most definitions, would change bias as well. Overall, we have arrived at an effective stalemate in the literature in which each group claims that they are properly determining bias, a metric with no consensus-based standard.

Pitfalls of bias calculation strategies have been extensively reviewed [82, 86-88], so we won't provide further analysis on the available models. Importantly, signaling bias is inherently a relative measure, so it is challenging to compare the bias levels of various compounds across studies. Bias quantification requires a separate dose response curve be determined empirically for each different effector, typically G protein and arrestin-3. This requires a unique assay for each effector, and the options available are favored or avoided for various reasons. These assays employ artificial systems, often with some degree of signal amplification. Additionally, because no assay is widely accepted as the standard, comparing results across methods is difficult. Once dose-response curves are generated, the relative activity of these two effectors must be compared to that elicited by a reference compound, the choice of which is critical for the intended impact of the study. This raises another challenge in comparing work across groups due to differing opinions on which reference compound will generate the most relevant bias calculation. When the reference compound is a peptide, as is often the case in opioid studies, its potency is particularly vulnerable to factors like storage and preparation. Because experimental methods and common-use definitions vary between labs, the same drug can easily be classified as biased in one case and unbiased in another. Beyond procedural challenges, real biological phenomena can also complicate how bias is appreciated. For example, in different cellular compartments within a single neuron there are differences in the ability of morphine to promote receptor endocytosis [89].

Because methods of bias calculation all suffer their own flaws and are therefore selected based on opinion and preference, we fear that these arguments lack a clear

endpoint. Rather than pursue the debate on how best to calculate bias, we suggest it would be more valuable to return to the original question of the role of bias in opioid side effects. One avenue that has been under-explored is how side effects are influenced by agonism that is balanced or arrestin-biased. Following the canonical arrestin-3 knock out results, we know of no lab other than our own that has explored balanced agonism in earnest, even for the purpose of supporting the hypothesis that biased agonists perform better. This is made more difficult by the paucity of balanced agonists, although methadone, a full agonist at G that robustly recruits arrestin-3 even without GRK overexpression, has been excluded from many of the analyses.

The recent review from Kolb et al. gives an excellent explanation of how bias can be defined relative to any compound of interest (benchmark bias), a physiologically dominant agonist (physiological bias), or a GPCR signaling equally to both effectors (pathway bias) [86]. In translational studies of opioids, our group is most concerned with physiological bias (bias measured relative to an endogenous agonist such as endorphin or enkephalin). This practice is common enough but has largely been employed with the specific motive of identifying molecules that are unlike the endogenous agonists in their signaling behaviors. It is likely that bias of any opioid varies across tissue and cell type, not just because levels of GRKs and arrestins vary, but because efficacy does too. For example, neurons that are tonically active will look more sensitive to inhibition by opioids than neurons that rarely fire. Our view is that in this new chapter of opioids research we should instead cast more light on how unbiased, or balanced, signaling could be an avenue to reduce the negative impacts of opioid use. We see this as the appropriate angle from

which to approach drug development because, while signaling activity must be measured *in vitro*, at least if we wish to screen many compounds, these drugs are ultimately intended for use in a complex organism. The system in which opioid drugs are intended to operate is calibrated to the signaling profile produced by its own endogenous agonists. It follows that a drug with a similar signaling profile could exploit the endogenous analgesia mechanism while minimally perturbing the state of homeostasis.

As we explain in Figure 6, we consider signaling to be balanced when the relationship between G and arrestin-3 is equal to that of the reference compound at the same relative dose. It is therefore possible for agonists of variable potencies to be balanced provided the relationship of the two effectors reflects that of the reference compound (Fig. 6A). If both the G protein and arrestin dose response curves are shifted the same amount relative to the reference curves, the compound is considered balanced. Bias can occur via a change in either potency or efficacy at either effector (Fig. 6B-E). This also means that it is theoretically possible for an agonist to be balanced at some doses and biased at others, or even G protein biased at some doses and arrestin biased at others (Fig. 6E). This is not merely hypothetical as some second-generation antipsychotics are arrestin biased, doing a better job of promoting endocytosis of their target receptors [32] and/or engaging arrestin-mediated signaling there [90].

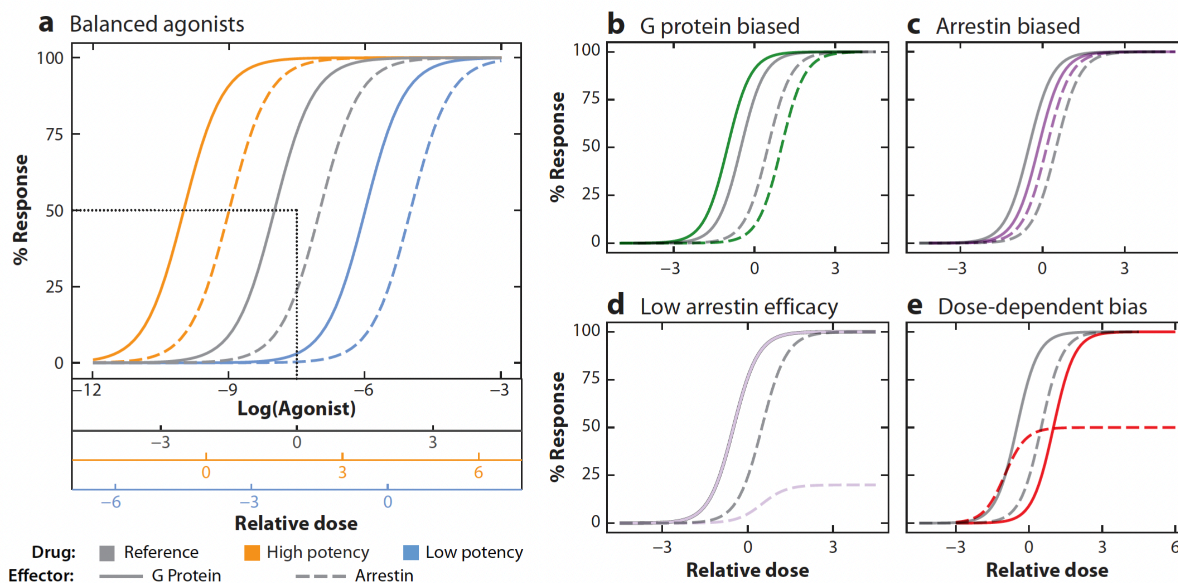

Figure 6: Examples of G protein and arrestin signaling profiles for balanced and biased agonists. (a) Agonists of low (blue) and high (orange) potency can be balanced when their G protein (solid lines) and arrestin (dashed lines) dose response curves are equivalently shifted from those of the reference compound (gray). Each agonist has a relative x axis determined by setting the midpoint between its two EC50 values as zero (demonstrated for the reference agonist with black dotted lines). Fully balanced agonists would therefore show superimposed curves if the x axes were aligned at their relative doses of zero. (b–d) Example drugs (colors) aligned to the reference compound (gray) at relative dose zero. (b) A G protein–biased drug will have a G protein curve that is more left shifted from its arrestin curve than the reference compound. (c) An arrestin-biased drug will have a G protein curve that is less left shifted from its arrestin curve than the reference compound. (d) A drug can be G protein biased if it is a partial agonist for arrestin, even when potency for both G and arrestin are identical to the reference compound (G protein curves are superimposed for the two compounds). (e) A drug that is more potent but less efficacious for arrestin recruitment will be arrestin biased at lower doses and G biased at higher doses.

This esteem for balanced agonism is not merely philosophical; it is well supported by several studies as described above. However, we need more studies and more balanced ligands to thoroughly test this hypothesis. This does not appear to be an impossible task. Most of the more recent ligands discovered have been ultra-biased because that was the intended product (TRV-130) or possibly because the structure used for virtual screening was not suited to identify balanced ligands, not because balanced ligands do not exist. It was recently shown using molecular dynamic simulations that the conformations

displayed by a methadone-occupied MOR are distinct from those for a morphine or TRV-130 occupied receptor [91] indicating that a different structure might identify additional balanced MOR ligands. A deeper dive into the literature shows that even in chemical series' designed to identify ultra-G-biased ligands, such as the herkinorins and recent SR series, more balanced ligands were identified (see compound 7B in [92] and SR14969 in [79]). Tianeptine, an antidepressant whose activity is mediated through MOR, may also be more balanced than existing opioids and shows reduced tolerance and dependence [93]. These hints, coupled with a recent natural products library screen that identified a balanced, albeit low potency, MOR agonist [94] suggest that novel balanced opioids are within reach.

2.6 Moving forward with a reconciled view of contradictory results

The relationship between MOR trafficking, tolerance, and dependence [95] has been overshadowed by the focus on defining signaling bias and reducing respiratory phenotypes. Tolerance is a highly consequential side effect of opioids given its underlying role in both dose escalation and dependence, common precursors to addiction. A responsible drug development strategy will therefore direct special scrutiny toward tolerance outcomes. We define tolerance as diminished response to a drug following previous exposure [38] (Fig. 4), a broad definition to encompass the myriad mechanisms that could be responsible for this effect. In a GPCR-mediated drug response, tolerance can be caused by changes to the receptors themselves or changes independent of receptors that occur prior to or downstream of agonist binding (Fig. 5). As discussed above, both increasing engagement with arrestins (in RMOR mice) and decreasing

engagement with arrestins (in arrestin-3 knockout mice and MOR 11S/T-A knock-in mice) enhances analgesia and reduces tolerance. It is in the best interest of the research community and the public to explain how both things can be true. Here, we propose a model wherein morphine tolerance is mediated both by partial desensitization of MORs and by homeostatic adaptations to prolonged G protein signaling that is poorly titrated by endocytosis and recycling (Fig. 7). We favor this model because it reconciles the observations made in wild type, arrestin-3 knockout, MOR 11S/T-A, and RMOR mice.

For many drugs, including opioids, it is common practice to treat receptor desensitization as a surrogate for tolerance. Many distinct mechanisms can cause desensitization of a receptor. In canonical GPCR signaling, c-tail phosphorylation partially disrupts the strength with which receptor couples to G protein. Recruitment of arrestins to these phosphorylated receptors causes more pronounced desensitization as G protein coupling is further impeded [96], and the process is completed by removal of the receptors from the surface via endocytosis. Following endocytosis, GPCRs are either recycled to the plasma membrane (resensitized) or targeted to the lysosome for degradation (downregulated). After desensitization and endocytosis, MORs are recycled to the plasma membrane, not degraded, and thereby resensitized [97] a process that appears to be altered following chronic morphine but not chronic methadone treatment [98]. Desensitization prevents receptors from initiating their signaling cascade and requires more drug to increase the number of occupied receptors, thus compensating for those rendered ineffective. Tolerance caused by desensitization is therefore similar

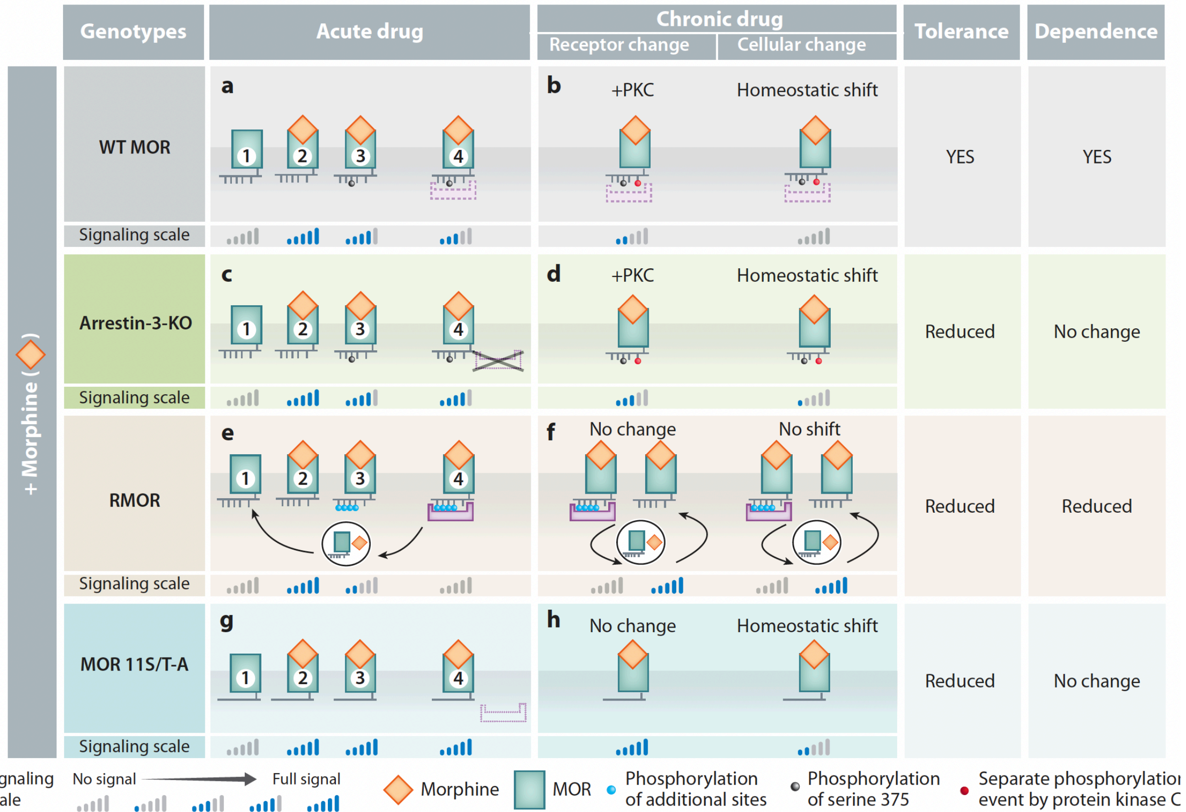

Figure 7: Model of desensitization mechanisms and cellular homeostatic shift in response to acute and chronic morphine in four genotypes of mice: WT, arrestin-3-KO, RMOR knock-in, and MOR 11S/T-A knock-in. (a,b) Signaling cycle of the WT MOR in response to acute morphine. (a) Morphine-occupied MOR is phosphorylated only on serine 375 (S375) ③, partially reducing signal. The partially phosphorylated MOR weakly recruits arrestin-3 ④, further reducing but not eliminating signal. This weak arrestin-3 recruitment is not sufficient to promote endocytosis and recycling so weakened receptor signaling persists. (b) Chronic morphine. The persistent signal from partially phosphorylated MORs triggers PKC phosphorylation, which further reduces (but does not eliminate) signaling. Homeostatic adaptations compensate further for persistent signaling. Both PKC phosphorylation and the homeostatic shift contribute to tolerance. (c) WT MOR response to acute morphine in arrestin-3-KO mice. As in WT mice (a), MOR is phosphorylated only on S375 ③, reducing signal, but no further reduction occurs via arrestin-3 ④. This explains the enhanced acute analgesia with morphine in arrestin-3-KO mice compared to WT. (d) Chronic morphine. The persistent signaling promotes PKC phosphorylation, further reducing signal. Homeostatic adaptations also compensate for the persistent signal, but because the receptors are more active due to no arrestin-3-mediated desensitization, tolerance is reduced compared to WT mice. (e) Signaling cycle of the RMOR in response to acute morphine. Morphine-occupied RMOR ② is completely phosphorylated ③, desensitizing signal. Arrestin-3 recruitment completes the desensitization ④, halting signaling and promoting endocytosis, recycling, and signaling restoration in response to ligand, initiating another cascade. Receptor recycling prevents the partial desensitization that occurs in WT, which explains the enhanced morphine analgesia in RMOR mice. (g) Signaling cycle of the MOR 11S/T-A in response to acute morphine. MOR 11S/T-A will not be phosphorylated ③ nor recruit arrestin ④. This explains the enhanced analgesia compared to WT mice. (h) Chronic morphine. The persistent signaling will promote homeostatic adaptations reducing signal and causing tolerance, but because receptors are more active due to no GRK or arrestin-3 desensitization, there will be less tolerance than WT mice. Abbreviations: GRK, G protein–coupled receptor kinase; KO, knockout; MOR, mu-opioid receptor; PKC, protein kinase C; RMOR, recycling MOR; WT, wild-type.

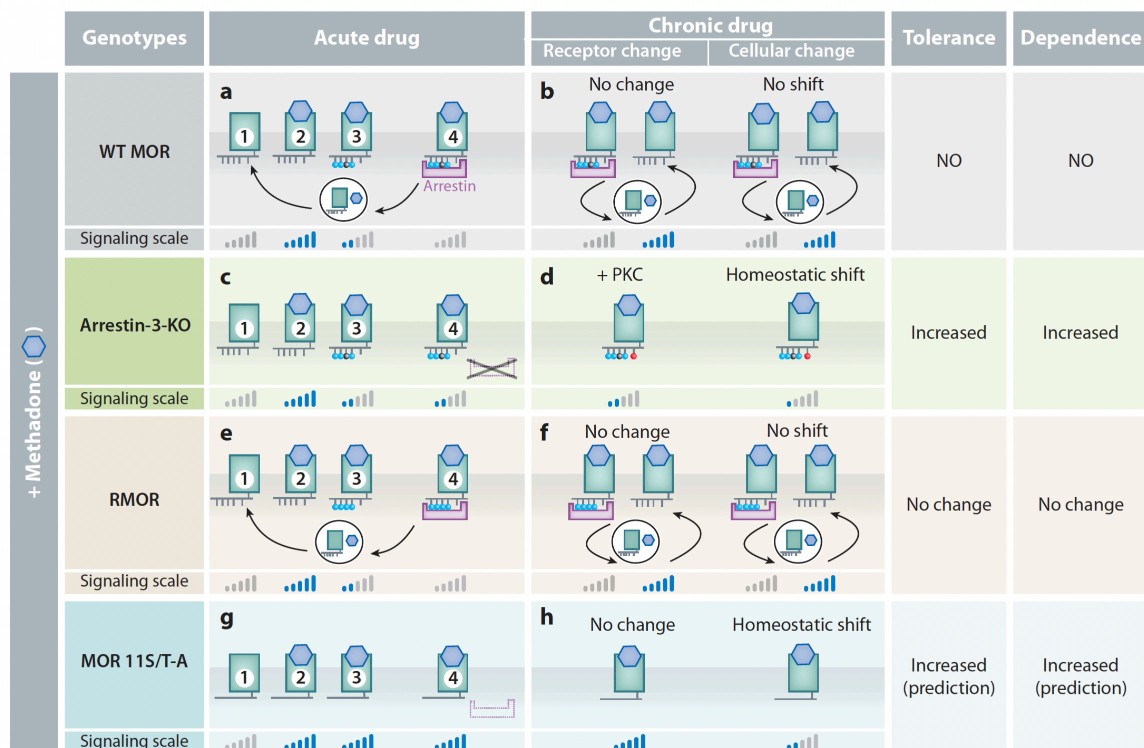

Figure 8: Model of desensitization mechanisms and cellular homeostatic shift in response to acute and chronic methadone in four genotypes of mice: WT, arrestin-3-KO, RMOR knock-in, and MOR 11S/T-A knock-in. (a,b) Responses to methadone. (a) Signaling cycle of the MOR in response to acute methadone: ① empty, ② methadone-occupied, ③ phosphorylated by GRK, and ④ arrestin-bound, endocytosed, and recycled. WT MORs are phosphorylated on four residues, desensitizing receptor signal ③. Arrestin-3 recruitment to phosphorylated receptors further desensitizes signal ④. Receptors are ① endocytosed and recycled, where they ② bind to ligand and initiate another signaling cascade. (b) Chronic methadone. While receptors constantly cycle on and off, signaling remains unchanged with no additional phosphorylation and no homeostatic shift. (c) Signaling cycle of the WT MOR in response to acute methadone in arrestin-3-KO mice. ③ WT MORs are phosphorylated on 4 residues, desensitizing receptor signal as in WT mice, but there is no further desensitization by arrestin-3 and no endocytosis. (d) Chronic methadone. Without arrestin-3 titration, homeostatic adaptations will occur due to the persistent low signal. This explains why arrestin-3-KO mice develop tolerance to methadone, but WT mice do not. (e,f) Signaling cycle of the RMOR in response to acute and chronic methadone resembles what occurs with morphine (Figure 7e,f). (g,h) These are predictions because this experiment has not been reported. (g) Signaling cycle of MOR 11S/T-A knock-in mice with acute methadone. MOR 11S/T-A will not be phosphorylated ③ nor recruit arrestin ④. (h) Chronic methadone. The persistent signaling will promote homeostatic adaptations, reducing signal and causing increased tolerance and dependence compared to that in WT. Abbreviations: GRK, G protein–coupled receptor kinase; KO, knockout; MOR, mu-opioid receptor; PKC, protein kinase C; RMOR, recycling MOR; WT, wild-type.

to tolerance due to a reduction in actual receptor number caused by receptor degradation/downregulation, not a typical fate of activated MORs [97]. Desensitization of

the MOR is variable across cell and tissue types, dependent on GRK and arrestin expression levels, and can differ based on which effector is measured [99]. For example, GIRK activation by the MOR in the periaqueductal gray is desensitized by enkephalins while MOR inhibition of transmitter release is not [100]. In these presynaptic terminals, MORs are still endocytosed following DAMGO treatment [101], indicating that the desensitization machinery remains intact. This does not translate to a change in apparent efficacy, presumably because there are enough spare receptors to amplify the signal. Functional response to morphine can also change at the cellular level even with no change in efficacy for G protein activation, a non-amplified signal [102].

Opioid drugs such as morphine cause incomplete phosphorylation of the receptor [61]. The poor arrestin-3 recruitment that follows is sufficient to cause partial desensitization of receptors on the membrane, especially with prolonged morphine treatment [103-107], but not to promote endocytosis and rapid resensitization. Balanced compounds such as met-enkephalin and DAMGO actually promote acute desensitization more completely than morphine, but the receptors are then rapidly recycled and resensitized [108]. Desensitization of GIRK activation by met-enkephalin occurs normally in arrestin-3 knockout mice [109] suggesting that phosphorylation alone may be sufficient for desensitization in some cases. It seems paradoxical that morphine produces desensitization and tolerance but not internalization, while enkephalin promotes both desensitization and internalization but not tolerance, until we consider the phosphorylation state of the MOR. While the degree of receptor phosphorylation affects the degree of arrestin-3 recruitment, it also likely affects the amount of arrestin-

independent (but phosphorylation-dependent) desensitization. As mentioned above, not all opioid ligands promote the same degree of MOR phosphorylation [61] (Fig. 7A, C). With enkephalin (or methadone), there is phosphorylation of the entire MOR barcode, which alone desensitizes MOR signaling and promotes arrestin-3 recruitment that shuts signaling down further. The receptor is then endocytosed, recycled and resensitized to agonist. Signaling at each individual receptor cycles between fully on and fully off but, because this is cyclical, the population always contains fully active receptors (Fig. 7A). With morphine, there is phosphorylation of the MOR only on serine 375 [61], which partially desensitizes MOR signaling and produces only weak arrestin-3 recruitment. Because the receptor is not endocytosed and recycled, signaling of each morphine-bound receptor is suspended in this partially but not fully desensitized state. This could explain why knockout of arrestin-3 increases morphine analgesia but does not alter methadone analgesia [110]. Although our understanding of how MORs are dephosphorylated is incomplete, ligands (e.g. DAMGO) that induce rapid phosphorylation and rapid endocytosis cause rapid dephosphorylation [111]. In contrast, ligands (e.g. SR-17018) that promote slow kinetics of phosphorylation [111], minimal arrestin recruitment [79] and diminished endocytosis [81] cause prolonged phosphorylation [111] and likely prolonged desensitization.

Repeated morphine treatment likely promotes additional receptor phosphorylation of partially desensitized MORs to further shut them down [103]. Rather than GRKs, this is seemingly mediated by protein kinase C (PKC) which has been shown to phosphorylate MORs in response to repeated (not acute) morphine but not DAMGO [112] (Fig. 7D).

Desensitization from chronic morphine treatment is also more persistent than desensitization from an acute dose [98], perhaps reflecting this additional phosphorylation event that is not removed through endocytosis and recycling or changes in the recycling rate through G protein activity [113] and/or cAMP levels [114]. There is therefore ample data suggesting that MOR desensitization by both GRKs and arrestins contributes to acute receptor desensitization and some evidence that tolerance to prolonged agonist engages both this and other mechanisms such as PKC. Deleting arrestin-3 would prevent a subset of the desensitization mechanisms and allow more MORs to remain active on the membrane (Fig. 7E,G), as would preventing any GRK or PKC phosphorylation (Fig. 7M,O). It is therefore not surprising that genotypes lacking MOR c-tail phosphorylation sites or with systemic arrestin-3 deletion show a potentiation of morphine analgesia and some protection from analgesic tolerance to repeated morphine. The partial desensitization produced by morphine, unreversed by endocytosis and resensitization, can also explain the enhanced morphine analgesia in RMOR mice (Fig. 7K). Because the RMOR undergoes complete phosphorylation, rapid endocytosis, and resensitization, partially desensitized receptors do not remain on the membrane acting as a sink for available ligand. In the original RMOR paper, this was the mechanism we proposed. In support of this hypothesis we demonstrated that a single morphine dose produced MOR desensitization in the brain stem of wild type but not RMOR mice [62].

Because tolerance takes days to develop *in vivo*, rapid receptor desensitization is likely not the sole cause. Tolerance can also result from homeostatic adaptations, even when receptor integrity is unaffected (Fig. 5). For example, changes in the availability of

second messengers downstream of transducer activation will change the system's response to a given concentration of agonist. Protracted MOR activation is known to increase levels of cAMP which opposes MOR-mediated adenylyl cyclase inhibition. Our work provides evidence that rapid desensitization, endocytosis, and recycling of the MOR prevents this homeostatic shift and also circumvents receptor desensitization by allowing for frequent turnover of active receptors (Fig. 7). This explains why there is tolerance to morphine but not methadone in wild type mice (Fig. 7A-D) and tolerance to neither morphine nor methadone in RMOR mice (Fig. 7I-L). Another group recently showed that morphine sensitivity can be restored by intrathecal DAMGO injection in the rat [115], further suggesting that receptor turnover is antagonistic to tolerance formation. This is similar to how rotation with methadone has previously been used in human medicine to achieve better pain control, although the mechanism was unknown [116]. In short, tolerance reduction and increased analgesia have both been observed when arrestin-3 activity is removed, and when it is enhanced. We view these seemingly contradictory observations as evidence that both the prevention of receptor desensitization, and the enhancement of resensitization can result in similar effects when measured at the level of *in vivo* drug responses.

Desensitized receptors can contribute to tolerance by lessening the signaling effect of an agonist when it is present. However, receptor desensitization alone cannot account for the opioid withdrawal effect. While tolerance and analgesia are both determined in the presence of drug, dependence must be measured in the drug's absence. We define dependence as behavioral effects that are not present in the naïve animal or while the

drug is being given at sufficient doses. We refer to this battery of effects as withdrawal, and it is precipitated either by cessation of opioid administration, or by giving a competitive antagonist such as naloxone [38] (Fig. 4). The onset of withdrawal symptoms when a drug is removed directly implies that these symptoms were being suppressed in the drug's presence, a task that a silent/desensitized receptor would not accomplish. The appearance of dependence is evidence that mechanisms downstream of the receptor are working in opposition to the opioid signaling cascade. We propose that adaptations such as cAMP superactivation contribute to tolerance by compensating for signaling that comes from receptors that are still functional and not properly titrated through endocytosis. They also cause dependence by inciting the cell's hyperactive state that results from agonist removal and subsequent silencing of those functional receptors. For example, we have demonstrated that inhibiting cAMP activity in the VTA during naloxone-precipitated opioid withdrawal prevents withdrawal symptoms, and RMOR mice do not show the cAMP dependent changes in VTA plasticity in response to morphine that wild type mice do [64]. Critically, RMOR mice show both reduced tolerance and reduced dependence, likely because both partial desensitization and homeostatic adaptations are absent in these mice. In contrast, only the partial desensitization is prevented in arrestin-3 knockout and MOR-11S/T-A mice, which explains why they still display dependence, in some cases exacerbated compared to wild type mice. By this model, balanced ligands should show reduced tolerance and dependence, as we see with morphine in the RMOR mice, while ultra-G-biased ligands should reduce only tolerance, as we see in the arrestin-3 knockout and MOR-11S/T-A mice. By extension agonists that are balanced in wild types

(like methadone) and do not produce tolerance and dependence should produce more tolerance and dependence in MOR-11S/T-A and arrestin-3 knockout mice compared to wild type mice, since the homeostatic shift will be engaged (Fig. 7N). Given the prominent role that dependence plays in the experiential side of drug abuse and the transition from therapeutic drug taking to the behavioral components of OUD, its clinical relevance should not be ignored. For these reasons we endorse a research goal that prioritizes a signaling profile that mimics that of endogenous agonists, preventing both tolerance and dependence.

2.7 Opioids research must prioritize human outcomes

In closing, we are compelled to point out that the search for novel G-biased ligands has transpired with remarkably little attention to how tolerance and abuse-liability impede the utility of clinical opioids. This push discounted reports that arrestin-3 knockout mice show enhanced morphine reward and no reduction in morphine dependence in favor of the goal to reduce OIRD. Furthermore, the observed respiratory effects of these new agonists were often conflated with other side effects like tolerance, reward, and dependence, fueling the drive for ultra-G-biased ligands at the expense of any other approach. While none of the common side effects of these drugs are trivial when considering their impact in individual scenarios, we should prioritize these effects based on their relevance in the greater public health context. The respiratory danger posed by opioid drugs is highly relevant once dose escalation and/or the cycle of opioid abuse begins but largely irrelevant when they are given at standard clinical doses under the direct supervision of a physician, as is the case with all novel therapeutics. We know that

lethal overdose frequently involves illicitly obtained substances, the possession of which is largely affected by availability and affordability. Therefore, the context in which OIRD is the most influential opioid side effect is unlikely to be directly impacted by the introduction of a new drug to the market as existing systems of illicit drug access will remain intact. Given our current understanding of how frequently illicit drug use is precipitated by medical use of addictive substances, we are obligated to be vigilant against the manufacture and distribution of new drugs with potentially increased risk for tolerance, dependence, and abuse behavior. An agonist with minimal respiratory effect but high dependence risk is not an improvement over the current options if it ultimately increases the number of people at risk for lethal overdose. Furthermore, OUD still carries deep social repercussions resembling those seen in less acutely lethal substance use disorders. For these reasons we believe it is critical to center tolerance and dependence prevention in the push for next-generation analgesics.

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Chapter 3 Deletion of arrestin-3 does not improve compulsive drug-seeking behavior in a longitudinal paradigm of oral morphine self-administration

3.1 Introduction

Opioids are powerful analgesic drugs that remain essential for the treatment of severe pain. Despite their therapeutic utility, opioid use can precipitate opioid use disorder (OUD). While most individuals who take opioids do not develop an OUD, over 2% of Americans age 12 and older meet the OUD diagnostic criteria [1] driving a major public health crisis, particularly with accidental overdose. Despite significant research efforts and billions of dollars invested, the development of an opioid with reduced abuse liability has been ultimately unsuccessful [2].This lack of success can be attributed in part to an incomplete understanding of how opioid signaling contributes to the physiological and behavioral components of OUD.

Opioid analgesia is primarily mediated by activation of the *μ*-opioid receptor (MOR), a G protein-coupled receptor (GPCR) [3]. 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) [4, 5] and recruitment of the arrestin-3 (β-arrestin-2) effector to the phosphorylated receptor [6]. Arrestin-3 recruitment not only uncouples MOR from its G protein but also promotes MOR endocytosis [7, 8]. Endocytosed MORs are then dephosphorylated and recycled to the plasma membrane where they can bind ligand and initiate another cycle of signal transduction [9, 10]. Activation of the MOR by opioid drugs, including morphine and all its

derivatives, promotes G protein signaling like endogenous ligands. However, morphine-activated receptors only weakly engage the GRK and arrestin-3 effectors [4, 11, 12]. This is because the morphine-activated MOR is phosphorylated on only one of the four residues [5] that are phosphorylated when the receptor is activated by an endogenous opioid. To denote this difference in MOR signaling by peptide or morphine occupied receptors, we refer to endogenous opioid peptides as balanced ligands: those that potently engage both the G protein and arrestin effectors. Small molecule opioid drugs are more biased: they more strongly engage G protein signaling in many cell types.

The impacts of biased and balanced signaling on the effect/side effect profile of opioid analgesics has been interrogated since the original discovery that morphine does not promote significant MOR endocytosis [13, 14]. Decades later, there remains little consensus on the role of arrestin-3 recruitment in opioid side effects because both eliminating arrestin-3 recruitment and enhancing arrestin-3 recruitment reduces some of the side effects of morphine and strengthens its analgesic effects. Mice without the arrestin-3 gene (Arr3-KO) were reported to show increased analgesia [15], reduced tolerance [16], and reduced respiratory depression and constipation [17] in response to morphine compared to wild type (WT) mice. Likewise, knock-in mice where the MOR is replaced by a mutant receptor which cannot be phosphorylated by GRKs (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 [18]. These data would suggest that removing arrestin-3 engagement improves analgesic utility. However, mice with a chimeric MOR that is an improved substrate for GRKs and have enhanced arrestin-

3 recruitment (RMOR mice, for recycling MOR) also show enhanced analgesia [19] and reduced analgesic tolerance to morphine with no change in respiratory depression [20]. In conditioned place preference (CPP) paradigms, both decreasing (Arr3-KO mice) [21] and increasing (RMOR knock-in mice) [22] arrestin-3 recruitment increases the potency of morphine reward. Finally, dependence, defined as physical and/or affective signs of distress upon the removal of drug, is another negative side effect of opioid use and a key component of OUDs. Both mouse lines deficient in arrestin-3 recruitment (Arr3-KO, MOR 11S/T-A) show intact or exacerbated morphine withdrawal signs, indicating that they still develop dependence [16, 18]. In contrast, RMOR mice show neither physical [19] nor affective [22] signs of dependence upon withdrawal from morphine. This battery of conflicting results has left the field divided on the best therapeutic strategy for new opioid drugs.

In humans, OUD is a syndrome defined by a constellation of phenotypes that include loss of control in drug-seeking behavior, craving, and relapse as well as physiological tolerance and dependence. We have previously reported a three-phase operant self-administration paradigm that models aspects of 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 demonstrate that some WT but no RMOR mice become compulsive drug-seekers with time [22]. However, it is not known how eliminating arrestin-3 impacts compulsive drug-seeking behavior. Since many of the side effects of opioids are improved with both the enhancement and the elimination of MOR-arrestin-3 interaction, its impact on drug-

seeking is difficult to predict. We utilized a version of our compulsive drug-seeking model in three genotypes: WT, Arr3-KO, and RMOR to determine how patterns of drug-seeking overtime were altered by increased and eliminated arrestin-3 activity at the MOR.

3.2 Methods

3.2.1 Mice

Mice of 3 genotypes were used in this study: 1) C57Bl/6 WT (n=20, 14 male, 6 female, 5 bred in-house and 15 purchased from the Jackson Laboratory) 2) RMOR [19] (n=15, 8 male, 7 female) bred in house, congenic >30 generations on C57Bl/6 and 3) Arr3-KO [15] (n=16, 7 male, 9 female) originally acquired from Dr. R. Lefkowitz (Duke University) [15] 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.

3.2.2 Determination of physical dependence to oral morphine

Following exposure to orally available morphine (see figure 1A,B), mice were assessed for physical dependence to morphine. Mice were injected subcutaneously with 5mg/kg naloxone and observed in clear plexiglass chambers for signs of withdrawal including jumping, wet-dog shakes, teeth-chattering, and paw tremors. A global withdrawal score was calculated as the sum of these behaviors.

3.2.3 Generation of oral dose-response curve to morphine

A dose-response curve to orally administrated morphine was determined using a radiant heat tail-flick assay (Tail-flick Analgesia Meter, Columbus Instruments. Columbus, OH). The light intensity was adjusted such that baseline latency (no drug present) to tail flick was 1.4-2.0 seconds. A maximum of three times the baseline latency (6.0 seconds) was used as a cutoff time to prevent tissue damage. A minimum of 5 independent subjects were tested for each dosing group. An oral gavage solution in sterile saline was prepared so that each subject received a maximum of 100μl when dosed by kilogram. Drug response latencies were measured 45 minutes following oral gavage of morphine solution. A non-linear fit equation in GraphPad Prism was used to determine the EC_{50} dose of oral morphine. Data are displayed as Analgesic Maximum Possible Effect (%MPE): 100*[(drug response latency–baseline latency)/(cutoff time– baseline latency)].

3.2.4 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. 2A). 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 progressed to the next FR schedule after they

obtained 20 rewards at each FR. 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: admittance into the study. 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.

3.2.5 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, the concentration of MS was 0.3 mg/mL in the first week and 0.5 mg/mL in the second week (Fig. 2Ai). After this, the concentration was increased to 0.75 mg/mL for the duration of the home cage drinking period. Mice had access to both the MS bottle and the water bottle 5 days per week and water only for the two days preceding each weekly operant session. MS and water bottles were weighed three times a week to monitor total morphine consumption.

3.2.6 Operant Oral Self-Administration Weekly Schedule

After saccharin training was completed, mice remained on the same weekly schedule for 16-19 weeks (Fig 2B). After two days of access to only water, mice were placed in the

operant box for a 30-minute session (peach bars, Fig 2Ai) that consisted of two distinct phases: a timeout period and a reinforcement period. The timeout 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 timeout period, which in our OUD model reflects futile drug-seeking. After the 5-minute timeout, the light above the active lever stopped blinking and remained on, initiating the start of the 25-minute reinforcement period. During this period, the first active lever press was rewarded by delivery of a 15 μl oral 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 variable interval reinforcement schedule were randomly selected from a 12-element Fleshler–Hoffman series to ensure all mice could access the same number of rewards [23]. In our OUD model, a variable interval schedule was chosen to capture 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 session. After the operant self-administration session, mice were returned to their home cage with *ad libitum* access to both water and morphine for the next 5 days followed by two days of water access. This weekly schedule was repeated for 16-19 weeks.

3.2.7 Extinction

Following 16-19 weeks of weekly operant self-administration, three 30-minute extinction sessions were conducted every day for a maximum of 12 days (Green bars,

Fig 2B). Extinction sessions were identical to the self-administration sessions except that lever presses on the active lever never led to a morphine reward or the associated tone and light cues during any part of the session. Each mouse was assigned an individual extinction criterion delineated as an active lever press daily session average below 20% of their weekly session average during the final three weeks of their self-administration phase or four or fewer active lever presses, whichever number was higher. Once this criterion was met, the mouse moved on to the next phase of the paradigm. Mice moved on to the next phase (abstinence) after 12 days of extinction training regardless of lever pressing behavior. Some mice therefore had more extinction sessions than others. All lever presses during these extinction sessions were automatically recorded. During the extinction phase, mice had access to only water (no morphine) in their home cage.

3.2.8 Abstinence and 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 2B). Following this abstinence period mice were returned to the operant box for a single operant session. This session consisted of a 5-minute timeout period identical to previous sessions. After this timeout period, the light over the active lever remained on and a single non-contingent (no lever press required) morphine reward was delivered at the port with the associated light and sound cues. After this single non-contingent reward delivery, the light remained on over the active lever, but no additional rewards or cues were delivered. During this session, all lever presses, all head port entries, and the latency to collect the non-contingent reward were recorded.

3.2.9 Calculation of compulsivity composite scores

Principle Coordinate Analysis and correlations analyses conducted in the R software packages factoextra (v 1.0.7) and corrplot (v 0.92) were used to identify measured behaviors through the paradigm indicative of drug abuse liability and that distinguish WT and RMOR mice from each other. A total of 16 measures from throughout the paradigm were selected to create a composite OUD/compulsivity score for each mouse (see Fig 4A for the variables used in the final score). The raw values for each mouse for each of these 16 measures were Z scored across the population of mice that completed the study (51 mice: 20 WT, 15 RMOR, 16 Arr3-KO). To give each phase equal weight when calculating the final score, a sub-score for each of the three phases (self-administration, extinction, reinstatement) was then created by averaging the Z scores of each behavioral measure in that phase for each mouse. The values for the operant self-administration phase came from the average of the final three weekly sessions for that mouse. The extinction values represented the average of the three sessions on each animal's first day of extinction. A final compulsivity score was then created by adding the self-administration, extinction, and reinstatement sub-scores for each mouse. The distribution of composite compulsivity scores of WT mice were bimodal (R software package mclust), thus we used the mean and interquartile standard deviation (IQD) of WT compulsivity scores to determine categorical assignments of compulsive or non-compulsive for the entire population. The IQD is defined as the standard deviation of values between Q1 and Q3. All mice with a composite score of 1 IQD or more over the mean score of WT mice were designated as compulsive.

3.2.10 Morphine Preference

On days 3-5 of the final week of the operant self-administration phase, 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 AS MS consumed (in mLs)/Total fluid consumed (in mLs).

3.2.11 Statistical Analyses

All statistics were conducted using R and the RStudio software except for Figure 1B & D, which were constructed in GraphPad Prism software. Statistical tests were chosen based on the distribution of data in each group. Normality of data was assessed using a Shapiro-Wilk test and data with a p-value greater than 0.05 was considered normal. One-way ANOVA or t-tests were used to compare differences between groups where assumptions for normality and homogeneity of variance were met. The Kruskal-Wallis was used when assumptions of normality were not met.

3.2.12 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.

3.3 Results

3.3.1 Oral morphine self-administration is sufficient to produce both analgesia and dependence.

To emulate human-like patterns of OUD in a rodent model, we developed a paradigm that allowed mice to engage in naturalistic drug-taking with substantial drug exposure but also yielded sufficient information to quantify motivated drug-seeking behavior. To

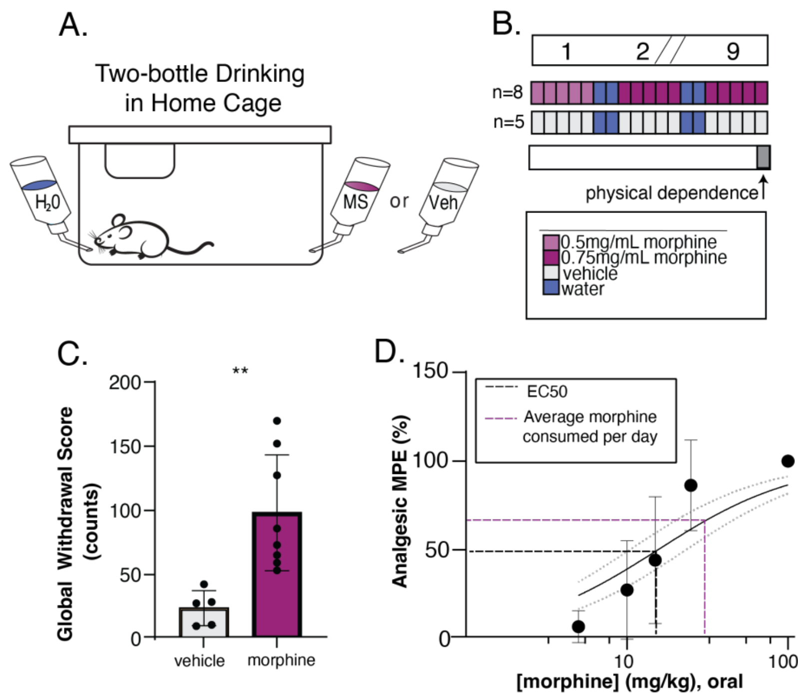
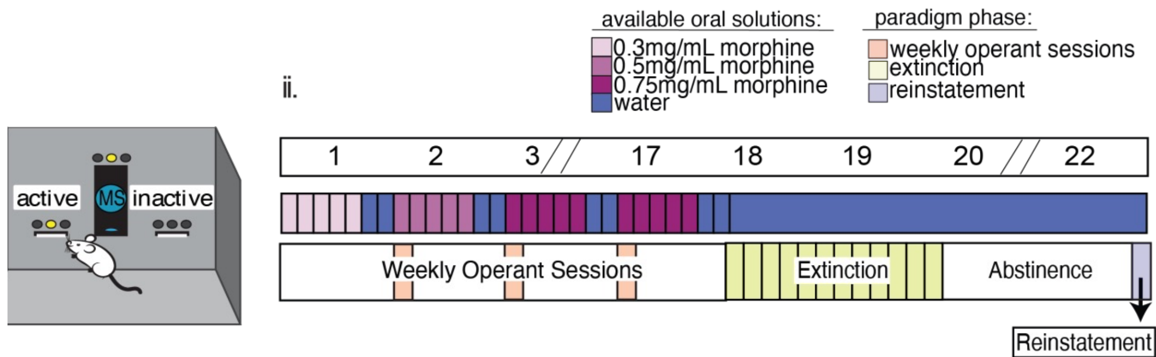


Figure 1: Oral consumption of morphine is sufficient to induce physical dependence and analgesia. A) Schematic of home cage setup with 24/7 water access and 24/5 morphine or 0.2% saccharin vehicle access. B) Experimental timeline to validate oral drinking exposure as a valid route of administration. Top bar shows time in weeks, where slashes indicate a repetition of previous weeks. Colored bars show available oral solutions in the home cage. Morphine at 0.5mg/mL (light purple) in 0.2% saccharin vehicle was available on the first week, then was increased to 0.75mg/mL (dark pink) in vehicle for the morphine drinking group (n=8). The vehicle solution alone (orange) was available to the saccharin drinking group (n=5). Mice had 24/7 access to water (blue), but two days a week the morphine or vehicle bottle was removed leaving the water bottle only. On the final day of exposure, naloxone precipitated withdrawal (physical dependence) was measured (gray bar). C) Physical dependence was assessed by injecting mice with 5mg/kg naloxone and calculating a Global Withdrawal Score for the subsequent 20-minute period (sum of jumps, wet dog shakes, teeth chatters, and paw tremors). The Global Withdrawal Score was significantly higher in morphine drinking mice as compared to their vehicle counterparts ($p = 0.0047$, two-tailed unpaired t-test). D) Analgesia was evaluated using a tail flick assay and a dose response curve was constructed to oral gavage of morphine (EC50 = 15.6) (black dotted line). The average amount of daily voluntary morphine consumption (31.3 mg/kg/day) (purple dotted line) is also visualized.

accomplish this, we utilized a combination of traditional operant self-administration and a variation of the two-bottle choice drinking task similar to a model we have described previously [22]. To validate that the paradigm provides sufficient drug exposure, we examined whether voluntary drinking on this schedule was sufficient to produce opioid dependence in WT C57Bl/6 mice. Following 9 weeks of home cage morphine drinking (Fig. 1A,B), we evaluated mice for common effects of opioid withdrawal precipitated by naloxone injection (5 mg/kg). Mice that had access to morphine in their home cage had significantly higher global withdrawal scores than those who had access only to the 0.2% saccharine vehicle solution ($p = 0.0047$, two-tailed unpaired t-test) (Fig. 1C). In a separate set of mice, we also confirmed that oral morphine at doses comparable to daily voluntary morphine consumption was sufficient to produce analgesia in a tail flick assay (Fig. 1D).

3.3.2 Deletion of arrestin-3 does not reduce drug-seeking behavior in a longitudinal OUD model.

The WT MOR recruits arrestin-3 very weakly in response to morphine activation when compared with the recruitment promoted by endorphins/enkephalins [24] (Fig. 2B, gray). To determine whether the degree of arrestin-3 recruitment to the MOR modulates drug-seeking, we employed our longitudinal mouse model of OUD and used two transgenic mouse lines with altered arrestin-3 recruitment. In Arr3-KO mice, the MORs have no ability to recruit arrestin-3 (Fig. 2B, orange). In RMOR mice, the receptor recruits arrestin-3 in response to both endorphin and morphine activation (Fig. 2B, teal).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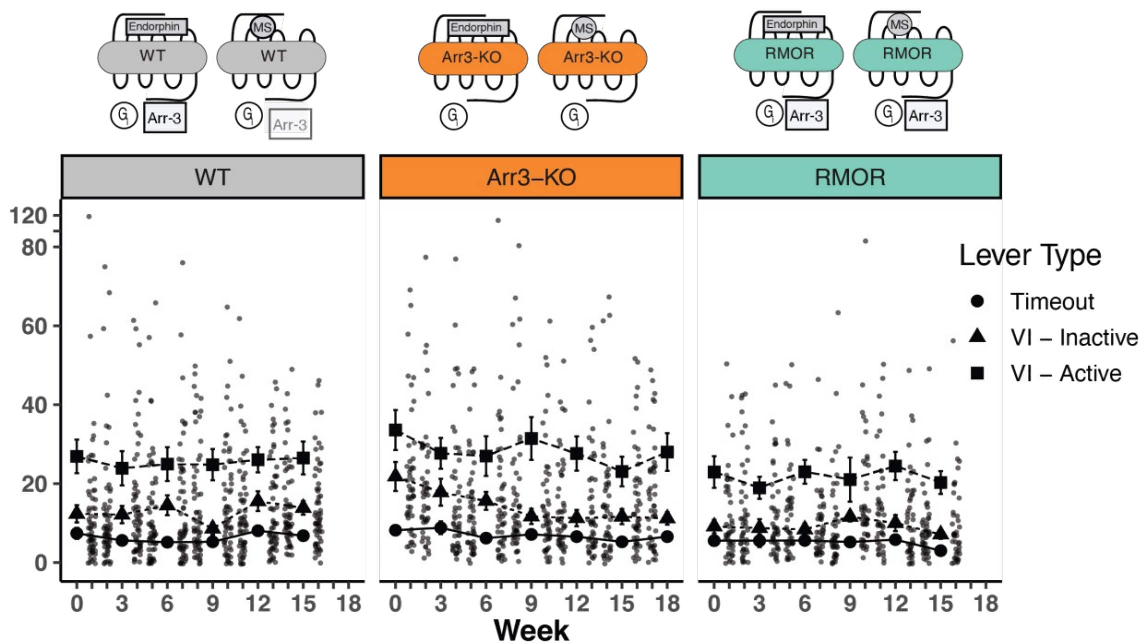
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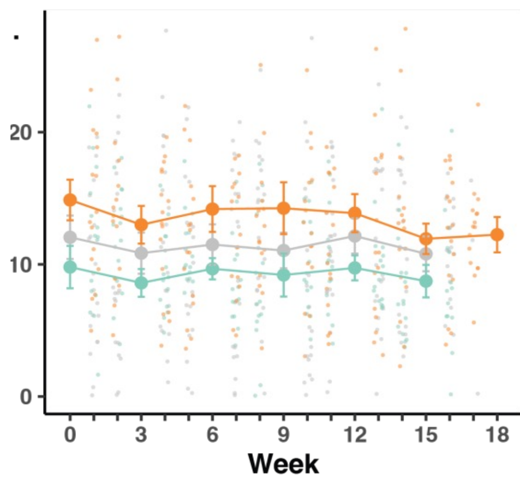
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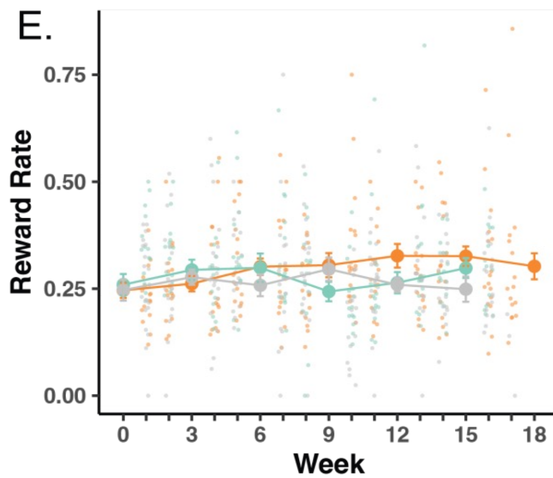
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Figure 2: Deletion of arrestin-3 does not reduce drug-seeking behavior in an operant self- administration task. A) Experimental paradigm for longitudinal model of OUD. i. Schematic of operant self-administration chamber where lever pressing resulted in delivery or denial of a morphine reward. ii. Experimental timeline. Top bar shows example weeks where slashes indicate a repetition of previous weeks. Middle bar shows oral MS availability in the home cage where blue bars represent water alone and increasing concentrations of morphine (0.3 mg/mL, 0.5 mg/mL, and 0.75 mg/mL morphine) are lightest to darkest purple (middle bar). Mice were able to drink morphine *ad libitum* in their home cage (Fig. 1A) for five days a week and water seven days a week during the self-administration phase of the paradigm. Bottom bar shows the three phases of the paradigm. Phase 1: 16-19 weeks of home cage drinking, with an operant self-administration session (peach bars) one day per week. Phase 2: Lever pressing behavior was extinguished in up to 12 extinction sessions (green bars). Phase 3: Cue-induced reinstatement (light purple bar) of lever pressing following a 14-day period of complete morphine abstinence. B) Schematic of MOR signaling in WT (gray), Arr3-KO (orange), and RMOR (teal) mice in response to morphine and the endogenous ligand, endorphin. Effectors include Gi/o/z protein (Gi, circle), Arrestin-3 (Arr3, square) C) Lever pressing behavior during operant self-administration phase in WT (gray), Arr3-KO (orange) and RMOR (teal) mice. Lever press counts are summarized (mean and standard error) for every three weeks of the self-administration phase with the distribution of individual subject counts displayed on the alternate weeks. Three types of lever press behaviors are described. Timeout (circles): any lever press that occurs in the first five minutes of a 30-minute session, Inactive (triangles): a press on an inactive lever during the final 25 minutes of a session, Active (squares): a press on an active lever during the final 25 minutes of a session. Only active lever presses could trigger reward delivery. D) Rewards collected during operant self-administration phase. Reward collection counts of WT, Arr3-KO, and RMOR (same colors as above) are summarized (mean and standard error) for every three weeks with the distribution of individual subject counts displayed on the alternate weeks. E) Reward rate during operant self-administration phase. Reward rate was calculated as rewards collected/total lever presses for each session. Session reward rates for WT, Arr3-KO, and RMOR (same colors as above) are summarized (mean and standard error) for every three weeks with the distribution of individual subject rates displayed on the alternate weeks.

To monitor the transition to compulsive drug-seeking and relapse as described previously [22], we implemented a paradigm which consisted of three separate stages: 1) Weekly Operant Self-administration 2) Extinction and 3) Reinstatement (Fig. 2Aii). Drug-seeking behavior was evaluated during each phase in an operant task (Fig. 2Ai) during which presses on an active lever may or may not yield an oral morphine reward on a variable interval reinforcement schedule. Mice were initially trained to press the lever for a saccharin reward and only mice who met the initial training criteria were advanced to the morphine-seeking task.

Mice in all three genotypes learned the task at equivalent rates and demonstrated a preference for the active lever over futile lever pressing (presses on an inactive lever or

on any lever during the initial timeout period of the session). Lever pressing activity was stable through many weeks of self-administration sessions, and there were no significant differences from average WT lever pressing behavior in RMOR or Arr3-KO mice (determined by one-way ANOVA with Tukey's multiple comparisons test) (Fig. 2C). On average, RMOR mice achieved fewer morphine rewards during operant sessions (Fig. 2D), but this was not statistically significant. When corrected for total lever pressing behavior, their reward rate was not different from the other two groups (Fig. 2E), reflecting that morphine is a more potent reinforcer in RMOR mice as previously reported [22].

Following the operant self-administration phase of the paradigm, mice were given extinction sessions three times daily in which cues and drug reward were no longer presented in response to active lever presses. Extinction sessions were ceased once a mouse met an individualized criteria determined as 20% of active lever pressing displayed during late operant sessions, or fewer than 4 lever presses in a session. Mice that reached 12 days of extinction training were automatically advanced to the next phase of the paradigm. The majority of mice extinguished their drug-seeking behavior within 12 days, but there was a significant effect of genotype on days to reach extinction ($p = 0.036$, $F = 3.566$, one-way ANOVA) as Arr3-KO mice took longer to reach extinction compared to the WT group ($p = 0.028$, Tukey's multiple comparisons test) (Fig. 3A). 7 out of 16 (43.75%) Arr3-KO mice did not reach their extinction criteria within 12 days, something that only occurred in 2 (13%) RMOR and 1 (5%) WT mouse.

After extinction, mice returned to their home cage for two weeks of abstinence with access to only water to drink. Following this abstinence period mice were returned to the

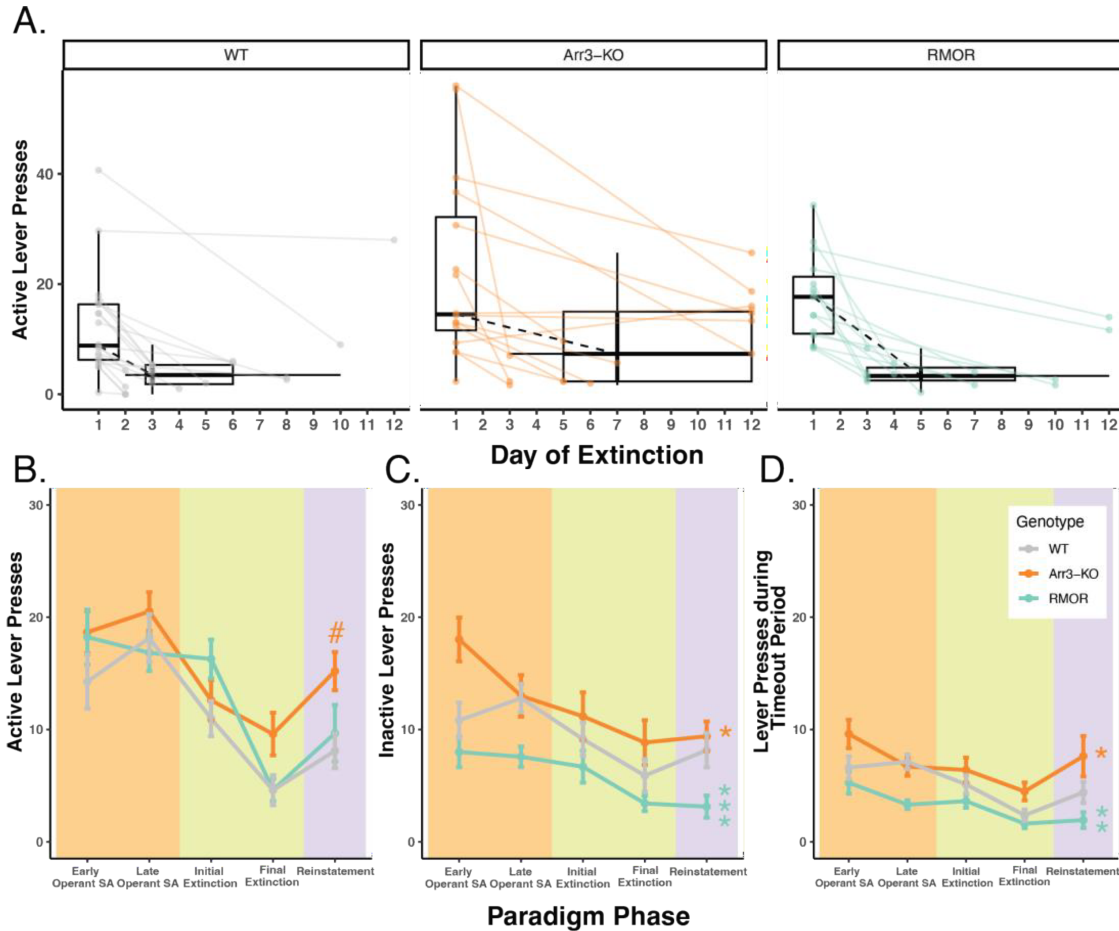


Figure 3: Deletion of arrestin-3 does not reduce drug-seeking behavior in a longitudinal model of OUD. A) Summary of active lever pressing during the extinction phase. Box plots and points represent the distribution of active lever press counts on the first day (Day 1) and final day (variable) of extinction in WT (gray), Arr3-KO (orange) and RMOR (teal) mice. Final day box plots also summarize the (horizontal) distribution of number of days to reach extinction which varied by mouse. Black dashed lines show the change in median lever press count between the first and median final day of extinction. Arr3-KO mice took significantly longer to reach extinction than WT mice ($p = 0.028$, One-way ANOVA with Tukey's multiple comparisons test). B) Active lever presses during each paradigm phase. Each phase of the paradigm, self- administration (peach), extinction (green), and reinstatement (purple), is denoted by background colors. Within the self-administration phase the lever presses from the first three weeks (early) and final three weeks (late) are summarized separately. Within the extinction phase, the lever presses from the initial (Day 1) and final (variable) are summarized separately. Mean and SEM are shown for WT (gray), Arr3-KO (orange) and RMOR (teal) mice. Genotype significantly affected active lever pressing across the paradigm ($p < 0.001$, Kruskal-Wallis test) Arr3-KO mice showed significantly more active lever pressing than WT and RMOR during the reinstatement phase ($p = 0.035$ & $p = 0.039$ respectively, Dunn test). # indicates significant difference from WT within individual phase. C) Inactive lever presses during each paradigm phase. Data are displayed according to the specifications of B. Genotype significantly affected inactive lever pressing across the paradigm ($p < 0.001$, Kruskal-Wallis test). RMOR ($p < 0.001$) and Arr3-KO ($p = 0.017$) mice displayed significantly different lever pressing than WT (Dunn test). * indicates significant difference from WT of all data across phases. D) Lever presses during the timeout period for each paradigm phase. Data are displayed according to the specifications of B. Genotype significantly affected inactive lever pressing across the paradigm ($p < 0.001$, Kruskal-Wallis test). RMOR ($p = 0.002$) and Arr3-KO ($p = 0.017$) mice displayed significantly different lever pressing than WT (Dunn test). * indicates significant difference from WT of all data across phases.

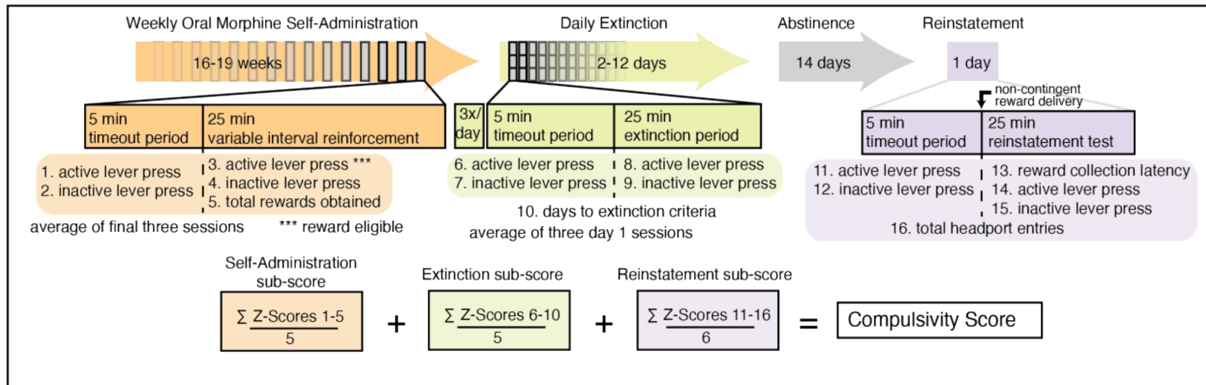
operant box for a single operant reinstatement session. This session was identical to a single 30-minute extinction session except mice received a single non-contingent morphine reward at the termination of the timeout period. Genotype significantly affected drug-seeking behavior during reinstatement ($p = 0.02$, Kruskal-Wallis test) as Arr3-KO mice displayed more active lever pressing than WT and RMOR groups ($p = 0.035$ & $p = 0.039$ respectively, Dunn test) (Fig. 3B). This is likely because several Arr3-KO mice did not effectively extinguish their drug-seeking behavior. A Kruskal-Wallis test did not reveal a significant genotype effect in active lever pressing on the final extinction day ($p = 0.068$), but Arr3-KO mice pressed more than other groups reflecting their lack of extinction. Overall futile lever pressing (inactive lever pressing or lever pressing during the timeout period) was significantly affected by genotype ($p < 0.001$ for both futile lever types, Kruskal-Wallis test). RMOR mice displayed significantly less inactive ($p < 0.001$) and timeout ($p = 0.002$) lever pressing than WT despite no significant difference in their active lever pressing ($p = 0.559$, Dunn test) (Fig. 3C,D). Conversely, Arr3-KO mice had slightly more futile lever pressing behaviors overall ($p = 0.017$ for both futile lever types, Dunn test) compared to WT mice, though this is partially driven by their increased tendency to press the inactive lever early in the self-administration phase. Overall, these data show that while the RMOR phenotype may offer some protection from compulsive drug-seeking behaviors in this model, arrestin-3 deletion does not offer improved outcomes after prolonged morphine exposure and may increase compulsive drug-seeking.

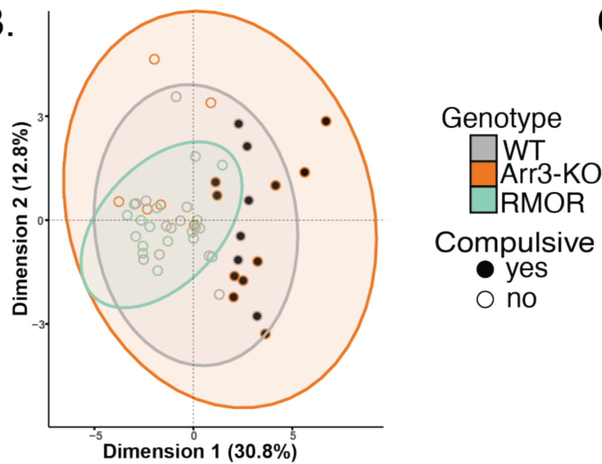
3.3.3 Arrestin-3 deletion does not improve compulsivity as defined by a behavioral composite score.

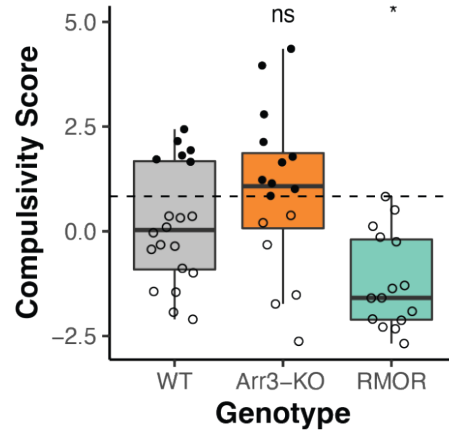
OUD is a complex diagnosis that involves a combination of behaviors and varies in its individual presentation. Because our experimental paradigm was designed to encapsulate several addiction-relevant behaviors, we considered a multi-variate analysis strategy. A Principal Coordinate Analysis (PCoA) of 16 behavioral measures across our entire operant paradigm (Fig. 4A) revealed that RMOR mice clustered tightly, whereas both WT and Arr3-KO mice had high variability across both dimensions (Fig 4B). We posited that this variability could reflect a bifurcation of phenotype in the WT and Arr3-KO groups in which a subset of mice adopt a compulsive behavior pattern just as only a subset of humans exposed to opioids develop OUD.

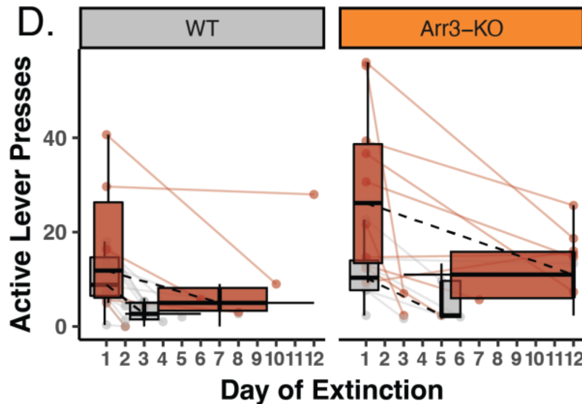
We calculated a composite score for each mouse that incorporated all 16 measures used in the PCoA (Fig 4A). Mice were designated as compulsive if their composite score fell above a threshold determined as one interquartile deviation above the mean composite score of the WT group (Fig. 4C). By these criteria, of the 20 WT mice, 6 (30%) were compulsive. Of the 16 Arr3-KO mice, 10 (62.5%) were compulsive. None of the 15 RMOR mice were compulsive, replicating what we have previously shown [22]. Comparison of composite compulsivity scores showed a significant genotype effect (Fig. 4C, $p = 0.001$, $F = 8.007$, one way ANOVA). In a Tukey's multiple comparisons test, Arr3-KO mice had no significant difference in compulsivity score from the WT group ($p = 0.262$), but WT and RMOR mice show a significant difference in compulsivity ($p = 0.031$) (Fig. 4C). These data confirm our previous work indicating that effective arrestin-3 engagement diminishes the liability for compulsive drug-seeking. Further, they suggest that preventing arrestin-3 engagement does not reduce compulsive drug-seeking and it

may even exacerbate it. This is a deviation from what we see with the physiological effects of analgesia and tolerance where both enhancement and elimination of the arrestin-3 pathway cause similar shifts. However, it aligns with the RMOR phenotype of reduced physiological and affective dependence that is absent in Arr3-KO mice.









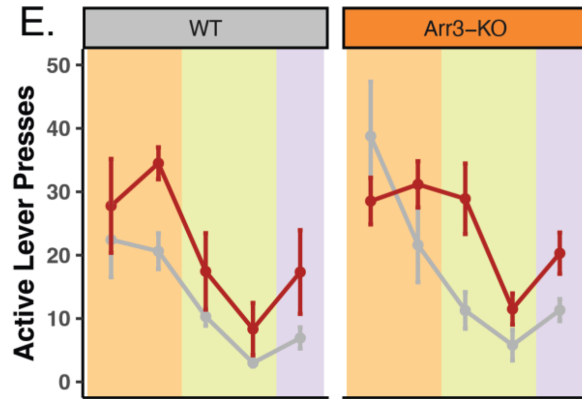


Figure 4: Arrestin-3 deletion does not improve compulsivity as defined by a behavioral composite score. A) Construction of composite behavioral score. Three paradigm phases, self-administration (peach), extinction (green), and reinstatement (purple, are displayed as a timeline with boxes below describing the details of a 30 minute task session. Variables included in the composite score are numbered 1-16 at their corresponding place in the paradigm timeline. The equation at the bottom of the panel displays the composite score calculation. B) Principal coordinate analysis was conducted using the 16 variables listed above. This revealed a tight cluster of RMOR (teal) mice while WT (gray) and Arr3-KO (orange) mice have more variable behavior. All but two of the compulsive mice (filled grey and orange circles) fall outside the RMOR cluster. C) Individual compulsivity scores for each mouse. Scores of compulsive mice (closed circles) were greater than one interquartile deviation above the mean composite score of WT mice. Non-compulsive mice (open circles) fell below this threshold. No RMOR mice were defined as compulsive. Scores of RMOR mice significantly differed from WT mice, but Arr3-KO mice did not in a one-way ANOVA with Tukey's multiple comparisons correction ($p = 0.031$ and $p = 0.262$, respectively). D) Active lever pressing during the extinction phase in compulsive and non-compulsive mice. Individual subject data (points and solid lines) from figure 3A are revisualized with compulsive (red) and non-compulsive (gray) mice summarized (box plots) as distinct groups. Black dashed lines show the change in median lever press count between the first and median final day of extinction for each group. RMOR mice are not shown as they do not have a subset of compulsive animals. E) Active lever presses during each paradigm phase in compulsive and non-compulsive mice. Lever pressing summary data from figure 3B is revisualized with compulsive (red) and non-compulsive (gray) mice summarized (mean and SEM) as distinct groups. RMOR mice are not shown as they do not have a subset of compulsive animals.

When we re-visualize the lever pressing behaviors after separating the mice into compulsive and non-compulsive groups, we observe a divergence of activity that is not apparent when we examined genotype differences. Compulsive mice take several more days to extinguish their lever-pressing behavior ($p = 0.012$, Kruskal-Wallis test) (Fig 4D). They also show an apparent escalation in drug-seeking through the self-administration phase that is not echoed by the non-compulsive group. This is apparent in the divergence of active lever pressing which is significantly higher in compulsive mice at the end of the self-administration phase ($p < 0.001$) despite there being no difference between the same mice at the outset of the phase ($p = 0.337$, Kruskal-Wallis test) (Fig 4E). This is not surprising, as these variables are contained in or derived from those within the composite scores used to assign the compulsivity threshold. It does, however, affirm the hypothesis that drug-seeking phenotypes may be more appropriately treated as bimodal than just

highly variable. This idea is bolstered by the tightly clustered variability of the RMOR mice, none of which were compulsive.

3.3.4 Compulsive drug-seeking behavior is independent of morphine consumption or preference.

The vast majority of the morphine consumption in our paradigm occurred during home cage drinking. Individual mice were highly variable in their weekly morphine consumption with a range of 2.09 to 11.1mgs consumed per week, on average. There was no significant difference in average morphine consumption ($p = 0.799$, Kruskal-Wallis test) between WT and Arr3-KO mice (Fig. 5A). There was no correlation in total morphine consumption and compulsivity score ($p = 0.57$, $R = -0.097$) (Fig 5B). There was also no significant difference in morphine consumption between compulsive and non-compulsive mice, (Fig. 5C, $p = 0.239$, Kruskal-Wallis test).

Because individuals with OUD often display a preference for opioid drugs over other sources of positive affect, we also assessed whether drug-seeking behavior was related to voluntary consumption of morphine (a drug reward) over saccharine (a naturalistic reward). During week 17 of our self-administration phase of the paradigm, we measured morphine and saccharine consumption in a traditional two bottle choice test over a four-hour period. There was no significant difference in preference for morphine versus saccharine between WT and Arr3-KO mice ($p = 0.156$, Kruskal-Wallis test) (Fig. 5D). Preference for morphine did not correlate with compulsivity score (Fig. 5E, $p = 0.61$, $R = -0.087$) and there was no significant difference in morphine preference between compulsive and non-compulsive mice ($p = 0.911$, Kruskal-Wallis test). These data indicate that morphine consumption and preference alone are not predictive of liability for

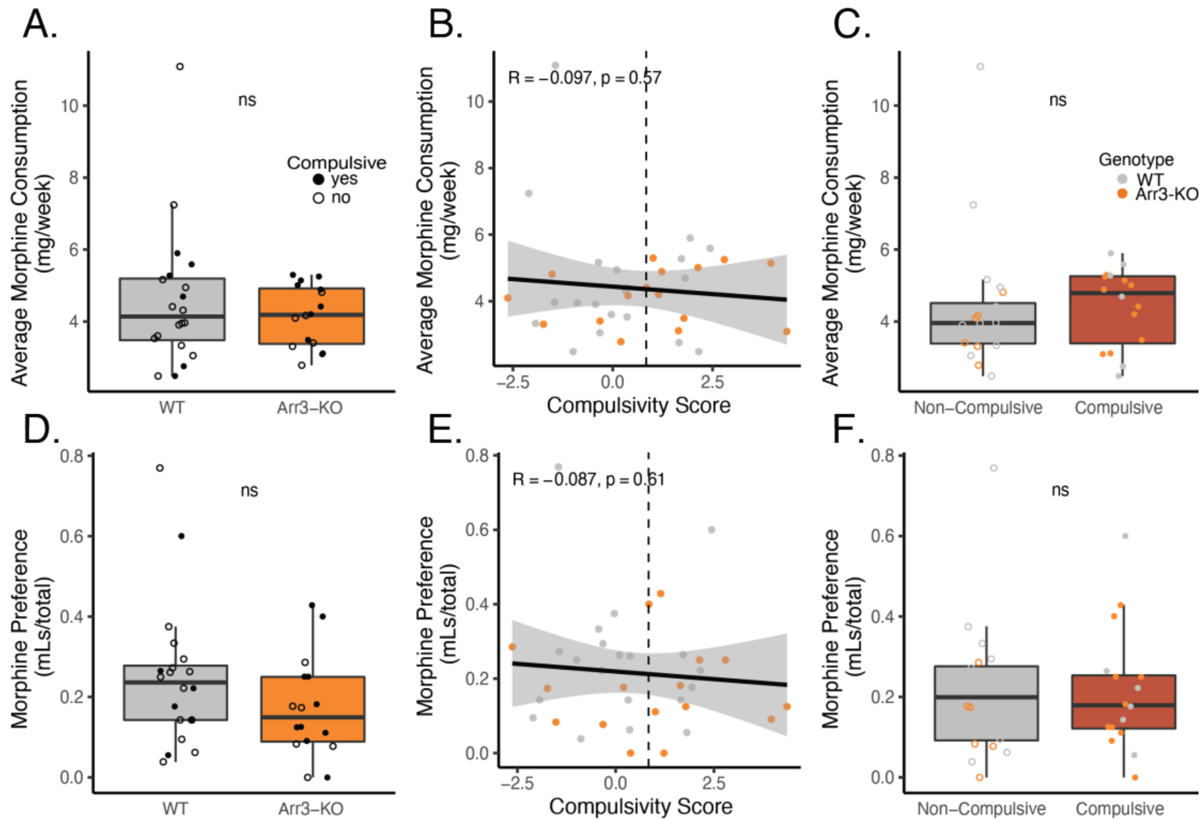


Figure 5: Morphine consumption or preference for morphine over saccharin does not predict compulsivity. A) Average weekly morphine consumption during the self-administration phase of the paradigm in WT (gray) in Arr3-KO (orange) mice. There is no significant difference between genotypes (p = 0.799, Kruskal-Wallis test). B) Average morphine consumption does not correlate with compulsivity score in a simple linear regression model (p = 0.57, R = -0.097). Vertical dashed line indicates compulsivity threshold score. C) Average morphine consumption does not differ between compulsive (red) and non-compulsive (gray) mice (p = 0.239, Kruskal-Wallis test). D) Preference for morphine over saccharin for WT (gray) and Arr3-KO (orange) mice. Preference for morphine in 0.2% saccharin vs 0.2% saccharin alone was measured on the final week of the self-administration paradigm in a 4-hour two-bottle choice test in the home cage. Preference (volume MS consumed/total volume consumed) did not vary significantly between genotypes (p = 0.156, Kruskal-Wallis test). E) Preference for morphine does not correlate with compulsivity score in a simple linear regression model (p = 0.61, R = -0.087). Vertical dashed line indicates compulsivity threshold score. F) Preference for morphine does not differ between compulsive and non-compulsive mice (p = 0.911, Kruskal-Wallis test).

compulsive drug-seeking behavior. This aligns with the realities of human drug use in which many individuals engage in medical or recreational opioid use without developing OUD.

3.4 Discussion

3.3.1 Arrestin-3-MOR activity does not cause or exacerbate compulsive drug-seeking

Here we use three genotypes of mice with different abilities to recruit arrestin-3 to the MOR to show that deletion of arrestin-3 does not protect against compulsive morphine-seeking in a mouse model of OUD. In a longitudinal paradigm that mimics human opioid consumption with a combination of *ad libitum* morphine access and contingent (motivated) drug-seeking, Arr3-KO mice displayed as much morphine-seeking behaviors as WT and RMOR mice. In addition to similar performance in the operant self-administration phase of the paradigm, WT and Arr3-KO mice consumed similar amounts of morphine and showed similar preference for morphine over naturalistic reward in non-contingent drug access contexts. When morphine reward was no longer available, Arr3-KO mice were slower to extinguish their drug-seeking behavior than the other groups. In fact, several Arr3-KO mice achieved the maximum number of extinction days and progressed through the paradigm without reaching their activity-based extinction criteria. This resistance to extinction may explain why the Arr3-KO mice had a stronger reinstatement effect. When we created a composite score to quantify compulsivity based on a multi-variate analysis of several behavioral outcomes, a subset of both WT and Arr3-KO mice were compulsive drug-seekers. In contrast, none of the RMOR knock-in mice exhibited drug-seeking behavior that reached the threshold for compulsivity (Fig. 4C).

These data make a clear case that loss of arrestin-3 activity does not protect against the behavioral components of OUD. While Arr3-KO mice were more vulnerable to developing some OUD-relevant behaviors in our paradigm, it is unclear whether this

means that low arrestin-3 engagement by opioid drugs increases their abuse liability. The Arr3-KO is a global knockout, and these effects could be influenced by the arrestin-3 pathway at any number of other receptors. Although it is clear that deletion of arrestin-3 does not improve outcomes in an OUD model, it is possible that engagement of the arrestin-3 pathway offers some protection from abuse liability of these drugs as we have previously reported [22]. No RMOR mice were classified as compulsive in this study nor do they develop analgesic tolerance to morphine under conditions where both WT [19, 20] and Arr3-KO [20] mice do. RMOR mice also do not show either physical [19] or affective [22] dependence whereas both Arr3-KO [16] and MOR 11S/T-A [18] mice show dependence at a similar or exacerbated level compared to WT. The development of tolerance and dependence presents major limitations to the clinical utility of opioids and are complimentary to the behavioral exemplars of abuse liability. These combined physiological and behavioral phenotypes in the RMOR mice justify a renewed interest in how arrestin-3 signaling might be exploited for opioid development strategies.

3.4.1 Opioid reward is an insufficient indicator of abuse liability

In our paradigm, which spanned several months, neither morphine consumption nor morphine preference was predictive of compulsive drug-seeking. Motivation to seek drug as measured by self-administration behavior also did not determine compulsive drug-seeking. Our data overall indicate that compulsive drug-seeking is not driven by opioid reward alone. This suggests that many of the behavioral assays, including simple operant responding, conditioned place preference, and consumption traditionally used as addiction proxies may not be predictive of actual liability to misuse drugs. This is

consistent with the observation that although morphine reward is enhanced in both RMOR [22] and Arr3-KO [21] mice compared to WT mice, RMOR mice do not transition to compulsive drug-seeking [22], while a subset of both Arr3-KO and WT mice do. As morphine is rewarding in all three of these genotypes [21, 22], these data indicate that future opioid drugs should be evaluated beyond their ability to produce reward with a more holistic understanding of abuse liability.

In humans, OUD is evaluated based on a diverse set of diagnostic criteria that encompass physiological, psychological, and social effects of opioid use [25]. Though it is impossible to recapitulate all these criteria in an animal model, more care could be taken to appreciate the heterogeneity of the disease. We attempted this with a multi-variate method that employs a PCoA and considers behaviors measured in multiple phases of an extended OUD paradigm. This allowed us to categorize animals into compulsivity groups based on a calculation that assigns equal importance to several behaviors that may or may not ultimately be relevant to the individual. Many models of substance use and misuse are well-established in the field, all of which have a role in unraveling the mechanisms of substance use disorders. Given the complexity of these disorders, it is in the interest of the field to adopt analytical approaches capable of simultaneously considering multiple animal behavioral outputs and how they may interact. We give one example here, but other techniques such as machine learning or meta-analyses would also be useful in evaluating these complex phenotypes.

3.4.2 Balanced agonism is an under-studied strategy with potential to improve opioid therapeutics

The differentiating characteristic of RMOR mice is that MOR signaling has been altered to reflect that of the endogenous opioids, as the MOR recruits arrestin-3 and is endocytosed and recycled in response to morphine [26]. This is not the case with the WT MOR which only recruits arrestin-3 when GRKs or arrestins are overexpressed [6, 11]. In neurons, opioid peptides, but not morphine, promote MOR endocytosis [13, 27], a consequence of arrestin recruitment. Likewise, *in vivo*, morphine administration also produces little endocytosis [26, 28] compared to opioid peptides [29-31]. Our data from RMOR and Arr3-KO mice imply that G protein-biased opioid ligands which do not engage arrestin-3 will not prevent abuse liability. This is a critical finding as opioid drug development has focused on the development of ultra-G-biased ligands for the past two decades. TRV-130 (Oliceridine) is one of these ligands and was FDA-approved in 2020—the first new opioid in 4 decades. This push to develop ultra-G-biased ligands followed reports that Arr3-KO mice show increased analgesia [15] and reduced tolerance [16] and respiratory depression [17] 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 [20, 32] and clinically, Oliceridine did not significantly reduce respiratory depression [33]. As no studies have assessed the abuse liability of the new ultra-biased ligands, our results indicate clinically relevant risks that should not be ignored. These findings, coupled with the recent reports on respiratory depression, indicate that ultra G-biased ligands are unlikely to improve on existing side effect risks. For all these reasons, we posit that more work is needed to assess the benefits of a signaling profile that resembles endogenous opioids [2].

We describe endogenous opioid signaling as balanced because it effectively engages both the G protein and arrestin-3 pathways. Recapitulating balanced signaling with exogenous ligands is immensely challenging. Categorizing ligands as balanced or biased depends on quantification of arrestin-3 recruitment and signaling to $G_{i/o/z}$ G protein effectors, techniques which are highly disputed and rife with caveats. This 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 [34-36].

The only clinically-utilized opioid drug that approaches a signaling balance comparable to endorphins and enkephalins is methadone [28]. No other existing balanced tool compounds have been tested *in vivo* because they have low potency [37], poor solubility [38], or were abandoned in favor of ultra G-biased ligands. In preclinical models, methadone produces less tolerance and less dependence than morphine [28]. Though it is rarely used as a first line analgesic in humans because of its highly variable half-life, a few controlled studies show reduced tolerance to methadone in humans (see review for studies within) [39]. However, methadone differs from morphine not only in pharmacokinetics but in many aspects of pharmacology [40]. This makes it difficult to say that bias is the primary factor in its reduced side-effect profile. It would be informative to examine methadone tolerance, dependence, and compulsive drug-seeking in a mouse model that cannot recruit arrestin-3 to the MOR, such as the MOR 11S/T-A knock-in mouse [18]. This effective conversion of methadone into a biased agonist would complement the findings from the RMOR mouse where morphine performs as a balanced agonist.

The phenomenon of the opioid epidemic demands multiple angles of attack. The phenotype of the RMOR mice gives hope that opioid agonists which provide both analgesia and reward without precipitating OUD could still be attainable. This goal remains vital as no alternative drugs exist for the treatment of severe pain. It is past time to expand our strategies in the areas of pharmacology and drug development and to meet this challenge with a tenacity that rivals that of this public health crisis.

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3.6 Author contributions

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

3.7 Funding and competing interests

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Chapter 4 Re-imagining opioids in a heterogeneous dopamine system

4.1 Introduction

4.1.1 Opioids disinhibit the dopaminergic neurons of the ventral midbrain

The effects of opioids are mediated by opioid receptors, all of which are Gi/o/z class GPCRs. These four receptors, the κ-opioid receptor, δ-opioid receptor, μ-opioid receptor (MOR) and nociceptin receptor, differ in their expression patterns [1, 2] and pharmacodynamic relationships with various opioid ligands. The MOR is responsible for the analgesic [3] and euphoric [4, 5] properties of opioid drugs. A key site of MOR activity in the central nervous system is the VTA, a mesencephalic region which, in combination with the substantia nigra pars compacta, produces much of the dopamine in the brain [6]. Dopamine is a monoamine neuromodulator involved in a diverse set of neural processes, most notably motor control, reinforcement learning, motivation, and reward. Delivery of dopamine signaling throughout the brain is facilitated by the long-range projections of these midbrain dopamine neurons.

The relationship between opioids and dopaminergic reward is canonically understood to be a disinhibition mechanism that enlists the GABAergic afferents of the VTA [7]. In the VTA, input from GABAergic afferent neurons provides tonic inhibition on the dopamine neurons and modulates dopamine release [8]. In this region, MOR expression is largely restricted to these GABAergic inhibitors [9]. As discussed in chapter 2 activated Gi-coupled GPCRs have a hyperpolarizing effect on neurons and can also inhibit vesicular fusion and transmitter release. The activation of MORs by opioid agonists

in the midbrain therefore reduces the tonic GABA release on dopamine neurons [7, 10-12]. GABA is an inhibitory neurotransmitter in the adult mammalian brain, so this reduction in GABA tone functions as a brake removal on VTA dopamine neurons. The mechanism of opioid reward is thought to rely on removing tonic inhibition of VTA dopamine neurons and leading to increased dopamine release in target regions, particularly the nucleus accumbens (NAc) [13, 14].

4.1.2 Chronic opioid exposure alters opioid-sensitive GABA neurons

Repeated exposure to drugs is often met with physiological changes designed to return a system to its homeostatic set-point and opioids are no exception. Tolerance is defined as a decline in the ability of a drug to produce the same physiological effect over time and is quantified by a rightward shift of a dose-response curve. Opioids also promote a state of dependence in which removal of the drug after a period elicits noxious and highly aversive symptoms. This altered physiological state that occurs when a drug is removed, called “withdrawal”, is related to tolerance but is a distinct opioid consequence that must be evaluated separately. Tolerance is a measured response to the addition of a drug and withdrawal is a response to that drug’s removal. Both of these states are observed in rodent models and manifest similarly to their presentation in humans [15].

Herein, we use “tolerance” and “withdrawal” to refer to physiological or behavior effects that can be measured at the level of the whole organism, but the term is also commonly used to describe changes that are measured on smaller scales. Chronic exposure to opioid drugs promotes such changes at molecular, cellular, and systems levels. The mechanisms of receptor desensitization and cellular cAMP changes are

discussed at length in chapter 2 and the various impacts of chronic opioid treatment on cellular physiology have been catalogued for decades [16]. The complexity of these phenomena is reflected by the fact that not all opioid drug effects exhibit tolerance. For example, locomotor effects of opioids undergo sensitization over time [17] and different drug exposure paradigms can sometimes determine whether tolerance or sensitization occurs [18].

In the VTA, chronic opioid exposure changes the opioid-sensitivity of presynaptic GABA neurons [19], particularly those in the rostromedial tegmental nucleus [11]. The homeostatic plasticity that befalls GABAergic afferents after repeated opioid administration has classically been demonstrated in ex vivo models of opioid withdrawal in which slices from opioid-dependent animals are subject to a loss in opioid signal from either drug washout or antagonist addition. Blockade of MOR signaling following chronic activation produces a strong increase in GABA release onto dopamine neurons in slices, a mechanism that is cAMP dependent [20, 21]. While we emphasized previously the distinction between tolerance and dependence (withdrawal) and their respective mechanistic requirements, the appearance of such a withdrawal effect in this circuit is an appropriate indicator that cellular tolerance was present and not driven entirely by receptor desensitization. An overall decrease in dopamine function is traditionally understood as a hallmark of tolerance and withdrawal from drugs of abuse [22]. However, this understanding is largely informed by studies that took place before the advent of modern genetic and optical tools that have revolutionized our ability to demystify neuronal function.

4.1.3 The mesolimbic circuit

The dopamine neurons of the VTA have several projection targets such as the medial prefrontal cortex (mPFC), basolateral amygdala and hippocampus [23], but the densest projections are those that target the nucleus accumbens (NAc). The NAc, often called the ventral striatum, is a large region of the basal forebrain largely populated by GABAergic medium spiny neurons (MSNs). It consists of an anatomically distinct core and shell as well as a mosaic patch-matrix organization in which the different compartments contain distinct cellular identities as well as afferent populations [24]. The MSNs of the NAc are often described as belonging to one of two subgroups: the D1 expressing and the D2 expressing MSNs based on which type of dopamine receptor they express. These subgroups differ in their projection targets in addition to their genetic profiles with the D1 MSNs projecting directly to the midbrain and the D2 MSNs projecting to pallidal structures within the basal ganglia [25]. This region has long been examined as a key center for pleasure and task motivation in the brain, though standard behavioral methods don't always make sufficient distinction between the two concepts [26].

The mesolimbic circuit is famously involved in the processing of reward prediction error (RPE): the disparity between the expected and actual outcomes of a decision or action. Since the discovery that dopamine neurons fire upon the delivery of unexpected rewards [27], an entire discipline of neuroscience has flourished with the intent to uncover the dopaminergic mechanisms behind reward prediction errors [28] and to further elucidate their role in learning, motivated behavior, and mental pathologies such as substance abuse [29]. Drugs of abuse are considered rewarding, though perhaps a more

thorough characterization is their ability to motivate individuals to seek them. The mechanism of reward in response to drugs of abuse is understood to involve increased dopamine release in the NAc [13, 30, 31]. Dopamine release from VTA neurons is inherently motivating [32], and all drugs that are popularly considered substances of abuse promote dopamine increases in the nucleus accumbens, albeit by different mechanisms. Opioids are no exception to this and cause an increase in dopamine release at VTA target sites [30, 31]. The disinhibition mechanism previously described is understood to drive the increased activity of VTA dopamine neurons. There is strong evidence to implicate VTA GABA neurons in mesolimbic reward as mice will press a lever to self-inhibit these neurons [31], mimicking the opioid disinhibition mechanism.

4.1.4 Chronic opioids and dopamine release

Most opioid studies have historically centered the acute (rewarding) effects of the drug, often relying on conditioned place-preference or self-administration paradigms to indicate the potential for drug misuse. Such studies are valuable but ignore the negative affective component associated with drug withdrawal and/or abstinence. Opioid dependence is often attributed to maladaptive changes within the dopaminergic reward circuitry. GABA terminals in the VTA are also activated by aversive stimuli and optogenetic stimulations of these terminals is sufficient to promote conditioned place aversion [33]. Repeated activation of this mesolimbic pathway by opioids leads to long-lasting plasticity in both VTA dopamine neurons and their downstream targets [34].

The previously discussed increase of GABA activity in the VTA of opioid-dependent mice is an appealing candidate mechanism for the aversive state of opioid withdrawal.

We have demonstrated that inhibiting cAMP activity in the VTA reduces both hyperactivity of GABA neurons in slices from morphine dependent mice and behavioral signs of morphine withdrawal [21]. This strengthens the case that opioid withdrawal involves homeostatic changes causing potentiation of VTA GABA inputs. By extension, it is also likely that this mechanism contributes to the loss of dopamine tone during withdrawal and the decreased potency of opioids that occurs over time with prolonged use. These consequences of prolonged drug exposure may underlie the development of opioid dependence and possibly the transition from voluntary to compulsive drug seeking.

Much of what we know about the effects of chronic opioids on the mesolimbic dopamine system stems from patch clamp studies which measured inhibitory activity on the dopamine neurons of the VTA. Our own experiments demonstrated that IPSCs in VTA dopamine neurons are either less sensitive to the inhibitory effects of DAMGO application or even potentiated by DAMGO application, an effect that does not occur in slices from opioid-naïve mice [19]. Work from another group offers one explanation for the emergence of these distinct populations of VTA dopamine neurons with the finding that IPSCs evoked from GABAergic afferents from the rostromedial tegmental nucleus are distinctly sensitive to this reduction in opioid sensitivity [11]. Beyond changes in how opioid delivery influences these circuits, there is also a body of work to show that GABAergic inputs to VTA dopamine neurons increase their output when in a state of opioid withdrawal [20, 21].

Based on our understanding of how GABAergic inhibition modulates the activity of dopamine neurons, it is reasonable to expect dopamine release activity to change in

response to opioid-induced changes in presynaptic GABA neurons. The well-characterized disinhibition mechanism of acute opioid exposure has long been considered the driving force behind the dopamine increase that follows opioid administration. Based on these factors we would hypothesize that basal dopamine levels would be reduced following chronic opioid treatment. We would also expect an opioid-induced dopamine increase to be reduced to reflect the decreased sensitivity of the neurons driving the disinhibition mechanism. Lasty, we would expect the precipitation of opioid withdrawal to cause a reduction in dopamine levels consistent with the increased inhibition that is precipitated by withdrawal in the VTA. Because previous studies of the VTA opioid circuit have not considered the heterogeneity or projection targets of dopamine neurons, these mechanisms are likely to apply differently to dopaminergic subpopulations. However, given the density of dopaminergic projections to the nucleus accumbens and suggested role of the mesolimbic pathway in the transition to compulsive drug use, it makes sense to first examine dopamine release in this region. Surprisingly, very few studies have tested these hypotheses to construct a more complete model of how dopamine release changes in animals that are tolerant or dependent on opioids due to chronic treatment.

In the early 1990s, Acquas and colleagues examined the effect of a 14-day morphine dose escalation on the basal levels of dopamine in the NAc as well as the effect of a low-dose morphine challenge on NAc dopamine release after morphine withdrawal [35, 36]. These experiments employed a transverse microdialysis probe that spanned the entire dorsal nucleus accumbens to include core and lateral shell regions. They found that

morphine abstinence after chronic treatment significantly reduced baseline dopamine tone, a change that began to recover after several days but was still below levels found in saline-treated rats one week post morphine cessation. They delivered a challenge dose of morphine and found that the morphine-induced dopamine increase was sensitized in rats following chronic treatment, an effect strongly dependent on how many days of abstinence had occurred. In a very similar microdialysis study [37], another group used a ten day dose-escalation paradigm and measured dopamine levels at 3 and again at 30 days following the end of morphine treatment. In this study, dopamine levels in the dorsal NAc core were sampled and they again found that morphine challenge after 3 days of abstinence causes a heightened dopamine increase in chronically treated rats. However, they did not detect changes in basal dopamine tone as the previous group did and the morphine sensitization effect was reduced but still detected 30 days post-treatment which the first group's results would not have suggested.

In a more comprehensive look at dopamine release during a chronic morphine study, Pothos et al used microdialysis to measure basal dopamine in the medial NAc and response to morphine injection [38]. These measurements were taken before and after rats received a week of daily morphine treatment. After a day of morphine abstinence, another microdialysis experiment was conducted to determine the effect of a naloxone injection. This study also did not report any difference in basal dopamine before and after daily morphine treatment. They also found no significant difference in the response to a morphine injection in mice before and after daily treatment. They did however capture a

response to naloxone-precipitated withdrawal in which dopamine levels decreased below baseline compared to naïve animals.

Another study in 2011 [39] used fast scan cyclic voltammetry to measure electrically-evoked dopamine release in the NAc medial shell of mice with chronically implanted morphine pellets. Dopamine release was significantly lower in the morphine-treated mice compared to sham animals even with the morphine pellets still in place. More recently, Lefevre and colleagues used fiber photometry to examine dopamine release activity in the NAc core before and after two contrasting chronic treatment paradigms [18]. They found that continuous treatment with an osmotic mini pump prevented some of the effects seen when morphine treatment is given with a single daily bolus.

It is challenging to synthesize these results into a cohesive model of how dopamine release is impacted by chronic opioid paradigms. First and foremost, there is the issue of anatomical differences between studies. The diversity of function contained within mesolimbic projections is now well appreciated given modern abilities to differentiate between projection targets. However, for most of the field's history, the VTA and NAc have largely been treated as homogenous regions. The studies mentioned above probe dopamine levels in distinct regions of the NAc, but no studies exist to date that have made rigorous comparison between regions with respect to a chronic morphine effect. Even one group that attempted to compare medial and lateral projecting activity with only acute heroin administration limited their comparison to the locations of cFos activated neurons [31]. Beyond probe location, these studies differ widely in their approaches. Some measure their outcomes in states of protracted abstinence while others use animals that

haven't had a period of spontaneous withdrawal. Some use separate control groups consisting of morphine-naïve animals while others take a within-subject approach and compare their outcome variables before and after drug treatment. They vary widely in their choice of dose and drug-administration schedule in addition to key methodological distinctions such as electrically-evoked vs drug-evoked dopamine release. None of this casts doubt on the validity of these studies but rather highlights the need to expand upon their findings with modern methods as there remains a massive amount of unexplored territory.

4.1.5 Dopaminergic heterogeneity

While dopaminergic neurons in the VTA are critically involved in both opioid reward and withdrawal [14, 38, 40, 41], midbrain dopamine neurons do not constitute a homogenous population. The cellular composition of the VTA is rich in phenotypic diversity and a variety of cell-types have been genetically and functionally identified [42-44]. VTA dopamine neurons project to several distinct regions including the NAc [23], and NAc sub-regions receive dopaminergic input from anatomically distinct sections within the VTA differently distributed along medial-lateral and anterior-posterior axes depending on their NAc targets [13]. In addition to this topographical organization, VTA dopamine neurons show potentiation under different reward and aversion conditions depending on their projection target [45], and also promote opposing behavioral responses [46]. The anatomical and functional complexity of these mesolimbic pathways has not been similarly considered when relating mesolimbic activity to drugs of abuse. The effects of opioids on VTA GABA release were determined with patch clamp recordings of

spontaneous and evoked inhibitory post-synaptic GABA currents (IPSCs) in VTA dopamine neurons by our lab and others, but previous studies have made no distinctions between the projection targets of the individual dopamine neurons. A handful of studies have implicated the medial portion of the NAc as the primary locus of drug reward [13, 31, 47], but little clarification has been made for the mechanisms of repeated drug use such as tolerance and dependence. Specifically, it is unclear if opioid reward and the aversive state of opioid withdrawal result from bidirectional modulation of the same circuit, or anatomically separable activity.

Not all dopamine neurons in the VTA react the same way to rewarding and aversive stimuli [48] and recent evidence suggests important differences in how NAc sub-nuclei respond under rewarding versus aversive conditions [49, 50]. De Jong and colleagues determined that the dopamine terminals in the ventromedial shell are active during aversive foot shocks and their associated cues but are also activated by rewarding sucrose stimuli provided that it is unexpected. In contrast, dopaminergic activity in the lateral shell is potentiated by reward, and cues for rewarding stimuli while aversive stimuli cause a depression of dopamine activity there. These findings raise many questions about drug reward and withdrawal in the mesolimbic system. It is unclear what sets drug reward apart from more ethological rewards such as sucrose and also remains to be seen if opioid withdrawal bears a functional resemblance to other aversive stimuli that involve the mesolimbic circuitry. This emphasizes how necessary it is to consider this anatomical separation in our evaluation of drug-induced states and presents an opportunity to

improve our understanding of mesolimbic opioid mechanisms by dissecting these different functional dopamine circuits.

4.1.6 Modernizing opioid pharmacology with neuroscience techniques

It is now clear that dopamine release has many functions in the midbrain, determined by the circuits in which it is embedded. The advent of sophisticated optical recording technologies has paved a new avenue for resolving the distinctions in these circuits and moved us beyond the cursory understanding of DA release in the NAc as a simple signal for reward and reward prediction errors. However, our understanding of where and how DA functions and circuits are coopted in the development of opioid dependence is largely unexplored. By exploiting genetically encoded molecular sensors and recording methods like fiber photometry, many of the previous barriers to studying pharmacological mechanisms in vivo are removed.

Fiber photometry is an optical imaging technique that allows local cellular activity to be monitored in sub-surface brain regions by collecting the output of a fluorescent sensor expressed in a region of interest. These sensors consist of a binding domain for a molecule of interest conjugated to a fluorescent protein such that the presence of that molecule causes the emission of fluorescent light. Fluorescent emission is relayed from an implanted fiber optic cannula through a cable that delivers intracranial signals to a recording system. Most fiber photometry utilizes a calcium sensor to monitor the firing of neurons using calcium influx as a proxy for action potentials. However, the last decade has brought the development of many sensors for fast-acting neurotransmitters, monoamine neuromodulators, neuropeptides, and hormones [51].

Several studies have now used fiber photometry to study opioids in the brain. In 2018, we saw the first examples of increased activity of dopamine neurons in response to heroin injections [31, 52]. Since then, fiber photometry has helped interrogate the role of these neurons in behavioral responses [53], the regulation of opioid-induced dopamine release [54], the opioid sensitivity of other cell types [55-57], and the importance of drug delivery patterns [18]. This list is non-exhaustive, and it can be a challenge to synthesize results across studies for multiple reasons. As discussed above, the anatomical placement of these probes determines the responses observed. Such responses can differ greatly even within short distances such as those between the core, dorsomedial and ventromedial shell of the NAc. Another concern is the use of a calcium sensor versus an extracellular measure such as dopamine release as we now know that these are not interchangeable proxies for each other [58].

The goal of the present study was to elucidate the role of different mesolimbic sub-circuits in opioid reward, tolerance, and withdrawal using the opposing anatomical framework suggested by De Jong et al [49]. We chose to measure dopamine release using a high dynamic range dopamine sensor (dLight 1.3b) [59] in either the lateral shell or the ventromedial shell of the NAc.

4.2 Methods

4.2.1 Mice

Male C57BL/6 mice were purchased from The Jackson Laboratory (Sacramento, CA). Mice acclimated to the housing conditions for at least 5 days after arriving and were housed in groups of 2-5under a standard 12/12-h light/dark cycle, with ambient

temperature set at 20 ^{o}C to 22 ^{o}C and food and water ad libitum. Initial surgeries were performed on mice aged 8–9 weeks and photometry experiments began at ~13 weeks of age.

4.2.2 Drug Preparation

All drugs were prepared in sterile physiological saline for injection volumes of 10 mL/kg (300 uL for a 30 gram mouse). Morphine sulfate (Mallinckrodt Pharmaceuticals) and naloxone hydrochloride (Sigma Aldrich) were prepared fresh from powder and used within seven days of dissolution. Brevital sodium (Par Pharmaceutical) was prepared fresh on day of use from 25 mg/mL stock aliquots stored at -20 ˚C. Veterinary carprofen (Rimadyl®) was diluted from 50 mg/mL sterile injectable solution to 0.5 mg/mL and used within 30 days of dilution.

4.2.3 Stereotaxic Surgeries

Mice were anesthetized with inhaled 2% isoflurane in oxygen and immobilized in a stereotaxic frame (Model 1900, Kopf Instruments) and injected with 100nL of AAV1-hSyn-dLight1.3b in either the medial (bregma: 1.50, lateral: 0.65, ventral: -4.75) or lateral (bregma: 1.00, lateral: 2.00, ventral: -4.50) shell of the nucleus accumbens. Virus was prepared by the UC Davis Molecular Construct and Packaging Core with a titer of ~10^{14} infectious units/mL. Virus was injected with a 33g stainless steel needle coupled to a 1 uL Hamilton syringe with polyethylene tubing backfilled with mineral oil at a rate of ~100nL/min with a microsyringe pump (World Precision Instruments). The needle was withdrawn slowly five minutes following the completion of virus infusion. Following virus infusion, a borosilicate optic fiber (400 um, NA: 0.66, Doric Lenses), was implanted at the

injection site and secured to the skull with Metabond dental cement (Parkell, Inc.) and a cement headcap was created to close the incision. At the time of implantation, a custom laser cut stainless steel headplate (manufactured by TEAM Prototyping Lab at UC Davis, design courtesy of the Stephan Lammel lab at UC Berkeley) was attached to the headcap to facilitate head-fixed photometry recordings. Mice were given carprofen (5 mg/kg, s.c.) at the time of surgery and every 24 hours for three days as a post-operative analgesic. No opioid analgesics were given until the time of photometry experiments in order to keep animals opioid-naïve.

4.2.4 Jugular Vein Catheterization

Four weeks following viral injection and fiber implantation, mice were anesthetized with inhaled 2% isoflurane in oxygen. A round tipped polyurethane catheter (Instech Labs) was inserted into the left jugular vein, trimmed to appropriate length for body size and attached to a compatible vascular access button (Instech Labs) that was then implanted subcutaneously between the shoulder blades. Mice were given carprofen (5 mg/kg, s.c.) at the time of surgery and every 24 hours for three days as a post-operative analgesic. Immediately following surgery, jugular catheters were flushed with physiological saline. Additional saline flushes were given every ~3 days and following i.v. drug injections to clear residual drug from the catheter. Mice were allowed to recover for 4-7 days prior to first photometry recordings. 4-24 hours prior to first photometry recording, catheter patency was tested with a bolus of brevital sodium 25 mg/kg i.v. (less if mouse was unconscious before completion of injection). Mice that were mobile within 5 seconds of injection bolus were removed from the study. Following the final photometry recording,

catheter patency was tested again. Mice were excluded from longitudinal analyses if catheter patency was lost before the conclusion of photometry experiments.

4.2.5 Fiber Photometry

For all fiber photometry recordings, mice were placed on a freely-rotating running wheel and head-fixed. Fiber photometry recordings were collected with a commercial fiber photometry system (Doric Lenses) that transmits LED light at 465 nm sinusoidally modulated at ~209 Hz and 405 nm sinusoidally modulated at ~308 Hz through a fluorescence mini-cube and into a fiber-optic cable that is coupled to the implanted fiber cannula on the mouse. Fluorescent emission travels back through the emission filter in the mini-cube and is directed onto a photoreceiver and then converted to digital signal that is recorded by the Doric software. The two channels are demodulated to separate dopamine and control (isosbestic) signals and decimated to 120 Hz for recording to disk. For each recording, 15 minutes of baseline was collected prior to i.v. injection and the recording continued for 15 minutes post-injection. Recording sessions that included two injections were completed in the same manner with the mouse remaining on the recording apparatus and a new baseline period beginning 15 minutes after the first injection.

4.2.6 Photometry Analysis

Preprocessing and analysis of photometry data was conducted using custom R scripts. To calculate dF/F, a simple linear model was used to regress values from the 465 nm channel onto the 405 nm values during baseline period of the recording (before drug injection). This model was then used to predict 465 nm values based on the 405 nm values for the entire recording. The dF/F was defined as (465 value – predicted 465

value)/predicted 465 value. dF/F signals were converted to z-scores using the mean and standard deviation from the baseline period: z-score = (dF/F – baseline mean)/baseline SD. A thirty second rolling average was applied to the z-scores to smooth data for group analysis. For peak analysis, data was pre-processed using a method adapted from Martianova et al. 2020 [60]. Briefly, an adaptive iteratively reweighted penalized least squares (airPLS) algorithm is used to dynamically detect the slope of each channel so it can be subtracted for a flattened trace that maintains high frequency signal changes. These adjusted signals are then standardized using the median and standard deviation of the baseline period: standardized signal = (signal – baseline median)/baseline SD. A robust linear model is used to predict the standardized 465 signal based on the standardized 405 and dF/F is calculated as the actual standardized 465 nm values minus the predicted values. Peak detection was performed on the standardized dF/F by calculating a rolling threshold defined as 2.91 times the median absolute deviation of a 30 second rolling window. Local maxima are then detected using the findpeaks() function from the pracma package with a minimum distance of 0.5 seconds between peaks. Local maxima that fall below the rolling threshold are considered background signal and excluded.

4.2.7 Daily Injections

Daily subcutaneous injections of morphine (10 mg/kg) or saline were given in the home cage between the hours of 10AM and 2PM. No subcutaneous injections were given on days when photometry recordings were happening.

4.2.8 Histology

Following the conclusion of the study, mice were anesthetized with isoflurane and transcardial perfusion was performed with 4% paraformaldehyde. Brains were extracted and post-fixed in 4% PFA for minimum 24 hours. 60 um coronal sections were cut and incubated overnight in a primary antibody solution with polyclonal chicken anti-GFP (ab13970, Abcam). The following day, slices were washed and stained for 1-4 hours with goat anti-chicken Alexa Fluor 488 secondary solution. Sections were mounted with glass coverslips using Vectashield Hardset with DAPI. Images were acquired using a Zeiss LSM 800 confocal microscope.

4.2.9 Statistical Analyses

Statistics were conducted in R with the RStudio software. Spontaneous transient data was analyzed with two-way ANOVA from the rstatix package. Early and late components of the recording were analyzed separately. To compare acute effects of IV morphine and saline injection, two-way ANOVA was used to determine the effects of drug injected and implant location. To compare the effects of chronic treatment, two-way ANOVA with repeated measures was used to assess the effects of daily drug treatment and recording day separately for each implant location and each drug response component.

4.3 Results

4.3.1 Morphine injection induces anatomically distinct dopamine increases in the medial and lateral NAc.

To compare the effects of morphine on dopamine release in the medial and lateral shell of the nucleus accumbens, we used an in vivo fiber photometry paradigm with IV morphine injection (Fig. 1A). Mice were injected with virus carrying the genetically encoded dopamine sensor dLight 1.3b in either the medial or lateral shell of the nucleus

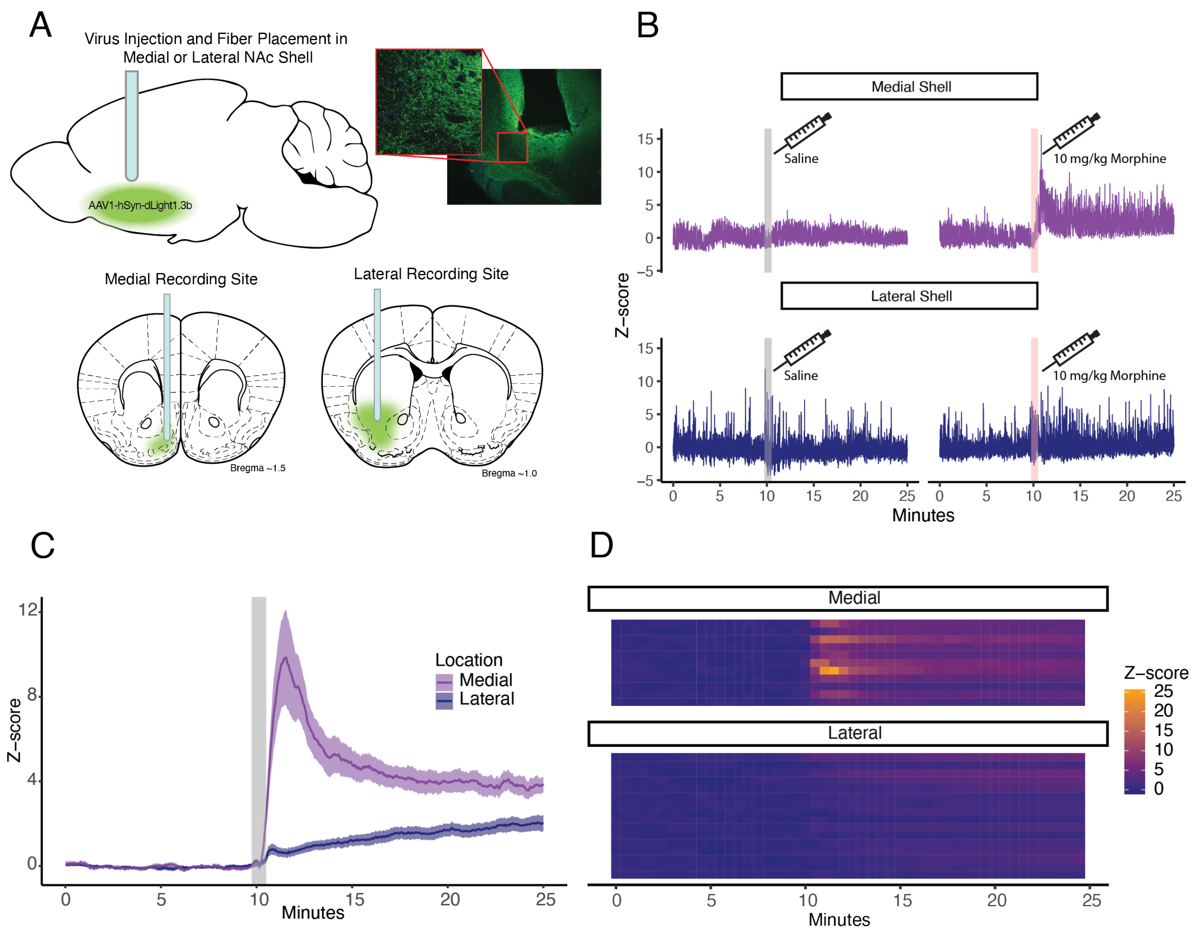

Figure 1: Morphine injection induces anatomically distinct dopamine increases in the medial and lateral NAc. A) Schematic of fiber photometry strategy. Optic fibers were implanted at the time of viral injection to allow expression of dLight1.3b in either the medial or lateral shell of the NAc. Approximate injection sites and fiber locations are shown in coronal sections. B) Representative traces of dopamine signal at baseline and following IV injection of morphine (right) or saline (left). C) Average dopamine response to IV morphine injection in the medial and lateral shell of the nucleus accumbens. Data has been smoothed over a 30 second rolling window. Shaded region reflects SEM. D) Heatmaps showing individual subject responses to IV morphine in 30 second time bins. Each row is a recording session for one mouse.

accumbens and a fiber optic cannula was implanted at the injection site. Four weeks after viral injection and fiber implantation, mice were implanted with jugular vein catheters to allow for instantaneous delivery of drug. Head-fixed fiber photometry recordings consisted of a 10-minute baseline period followed by an IV bolus of morphine (10 mg/kg) and an additional 15 minutes of recording. Individual subject recordings reveal both fast (sub-second) and slow (several minute) dopamine transient activity. Morphine injection caused

a large transient increase in overall dopamine signal in the medial shell of the nucleus accumbens characterized by a steep increase of the moving average followed by a short decay with a plateau above baseline after several minutes (Fig. 1C). In the lateral shell, minimal increase was observed in response to IV morphine with any increase appearing to ramp up over the 15-minute recording period.

4.3.2 Morphine injection alters spontaneous dopamine transient activity.

To examine the faster spontaneous dopamine signal (Fig. 2A), we used a different pre-processing algorithm to dynamically remove the slope of the recording [60] and then performed peak analysis to identify high pass dopamine transient events reflecting spontaneous dopamine activity. We first examined the patterns of peak count and peak prominence, defined as true peak height minus the local moving average, across the entire recording by visualizing them in one minute time bins (Fig. 2B) to see if the time course of peak changes followed that of the slower, morphine-induced dopamine changes (Fig. 1C). We found that fluctuations in peak prominence and frequency had a similar timecourse to the smoothed dopamine signal. Based on this timecourse, we chose to divide the morphine response into two distinct epochs for further analysis. The data was binned into the early response component: defined as the five minutes directly following injection, and the late response component: the subsequent ten minutes of the recording.

At baseline, there was a higher frequency of events in the lateral shell (0.149 peaks/s) than that of the medial shell (0.077 peaks/s) ($t = 7.773$, $p < 0.001$, Welch two sample t-test). The prominence of events was slightly higher at baseline in the medial (2.707) than the lateral (2.435) NAc shell ($t = -3.443$, $p = 0.001$). Frequency of spontaneous events

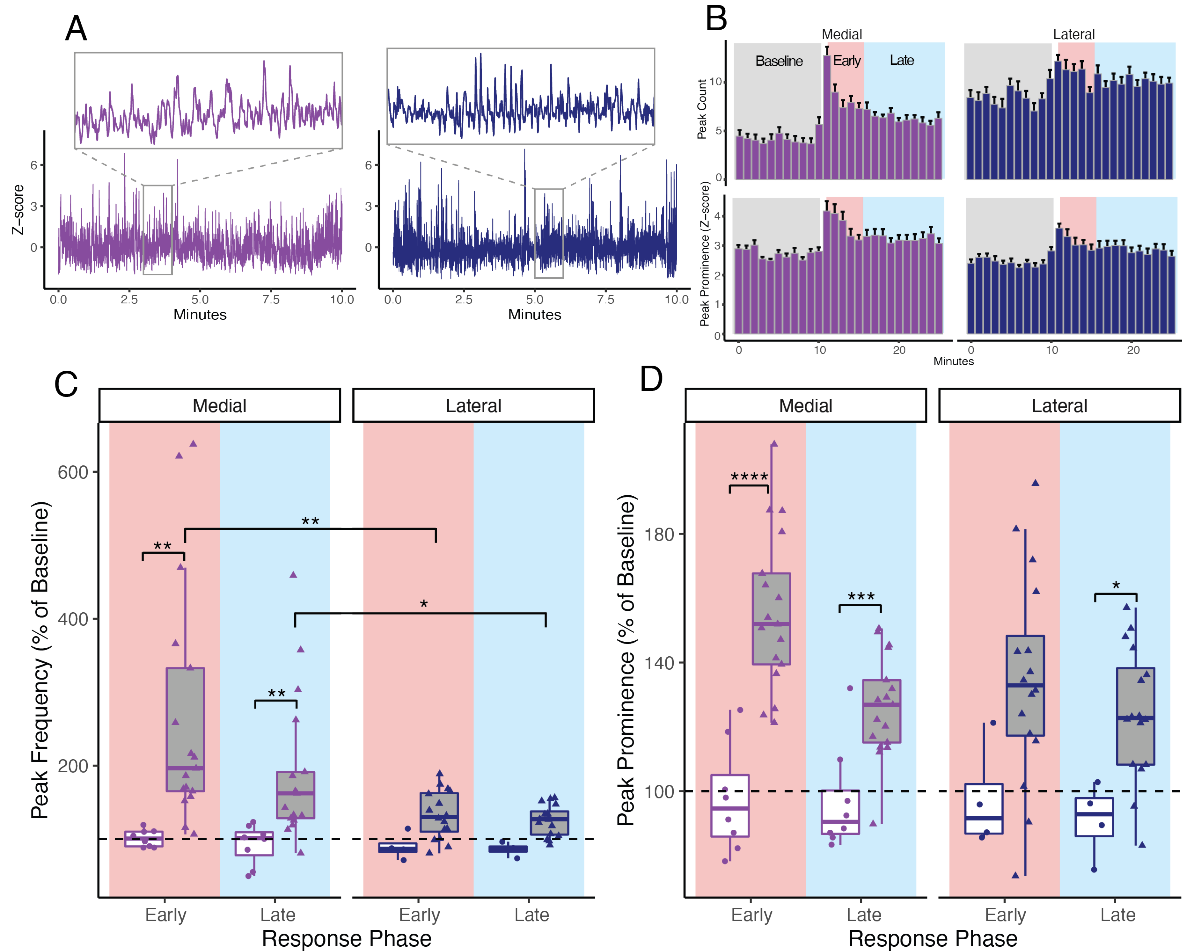

Figure 2: Morphine injection alters spontaneous dopamine transient activity. A) Representative traces showing spontaneous dopamine transient activity in the medial and lateral NAc shell at baseline. B) Peak count (top) and peak prominence (bottom) across the entire recording session. Data have been separated into one-minute bins to display average and standard error of each minute of the recording. C) Peak frequency in the medial and lateral NAc shell during early (red) component and late (blue) component response periods after IV injection of morphine or saline. Data is normalized to peak frequency during the pre-injection baseline period. Significant differences were found between medial morphine and saline response in early ($p = 0.004$) and late ($p = 0.007$) recording components and between the medial and lateral morphine response in the early ($p = 0.005$) and late ($p = 0.04$) component (Tukey's multiple comparisons test after two-way ANOVA). D) Peak prominence in the medial and lateral NAc shell during early component and late component response periods after IV injection of morphine or saline. Data is normalized to peak prominence during the pre-injection baseline period. Significant differences were found between medial morphine and saline response in early and late recording components ($p < 0.001$ for both) and between lateral morphine and saline response in the late ($p = 0.011$) component (Tukey's multiple comparisons test after two-way ANOVA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

was increased by IV morphine injection in the medial but not the lateral shell (Fig. 2C). A two-way ANOVA was performed to analyze the effect of implant location and morphine

injection. No interaction was found between the effects of implant location and morphine exposure during the early component ($F_{(1,44)} = 2.402$, $p = 0.128$) or the late component ($F_{(1,44)} = 0.013$, $p = 0.909$) response periods, but simple main effects analysis revealed a significant effect of morphine injection compared with saline in the early and late response components ($p = 0.002$ for both components). There was also a significant effect of implant location during early ($p = 0.006$) and late ($p = 0.037$) components. Event frequency in the medial shell was elevated (256% of baseline) directly following morphine injection, an effect that did not occur with IV saline injection ($p = 0.004$, Tukey's multiple comparisons test). During the late component of the morphine response, spontaneous frequency remained elevated (184% of baseline) and significantly higher than the saline response ($p = 0.007$, Tukey's multiple comparisons test) in the medial shell. This frequency increase was not detected in the lateral shell during the early or late components of the response and Tukey's multiple comparisons test revealed a significant difference in the effect of morphine injection on normalized frequency between the medial and lateral shell in both early ($p = 0.005$) and late ($p = 0.04$) response components.

Peak prominence was analyzed similarly to frequency. There was no interaction between the effect of implant location or treatment on the prominence of peaks in the early ($F_{(1,44)} = 1.655$, $p = 0.205$) or late ($F_{(1,44)} = 0.013$, $p = 0.909$) response components in a two-way ANOVA. Simple main effects showed an effect of morphine exposure in both response epochs ($p < 0.001$ for early and late components) but no significant effect of implant location. Peak prominence in the medial shell was significantly elevated by morphine injection (158% and 129% of baseline respectively in early and late

components) compared to saline in both response epochs ($p < 0.001$, Tukey's multiple comparisons test). In contrast to what was observed with spontaneous frequency, in the lateral shell peak prominence was increased relative to baseline by acute morphine injection, a change that reached significance relative to saline injection in the late response epoch (124% of baseline) ($p = 0.011$) but remained slightly below significance in the early component (135% of baseline) ($p = 0.065$).

4.3.3 Recurrent morphine treatment does not shift dopamine response to IV morphine in the NAc shell.

Chronic opioid treatment and opioid tolerance are hypothesized to reduce dopamine neuron activity based on changes in inhibitory pre-synaptic activity [11, 19]. To test the effect of chronic morphine on NAc dopamine release, we integrated our photometry recording protocol into a daily morphine treatment paradigm in which mice received six days of daily morphine (10 mg/kg, SC) or saline injection flanked by photometry recordings of IV morphine responses before and after this daily treatment paradigm (Fig. 3A). There was no difference detected in the response to morphine on day 7 compared to that observed on day 0 in mice that received daily morphine. This was true in both the medial and lateral NAc shell (Fig. 3B,C, left). Unexpectedly, in the medial shell of mice that received daily saline injections, there was a decreased response to morphine injection on day 7 compared to day 0 (Fig. 3B, right). The implication of this result is that daily morphine treatment precludes the diminished response to the secondary morphine injection observed in the saline treated mice.

Peak analysis revealed comparable frequency of spontaneous events between day 7 and day 0 for both early and late response components in medial and lateral shell

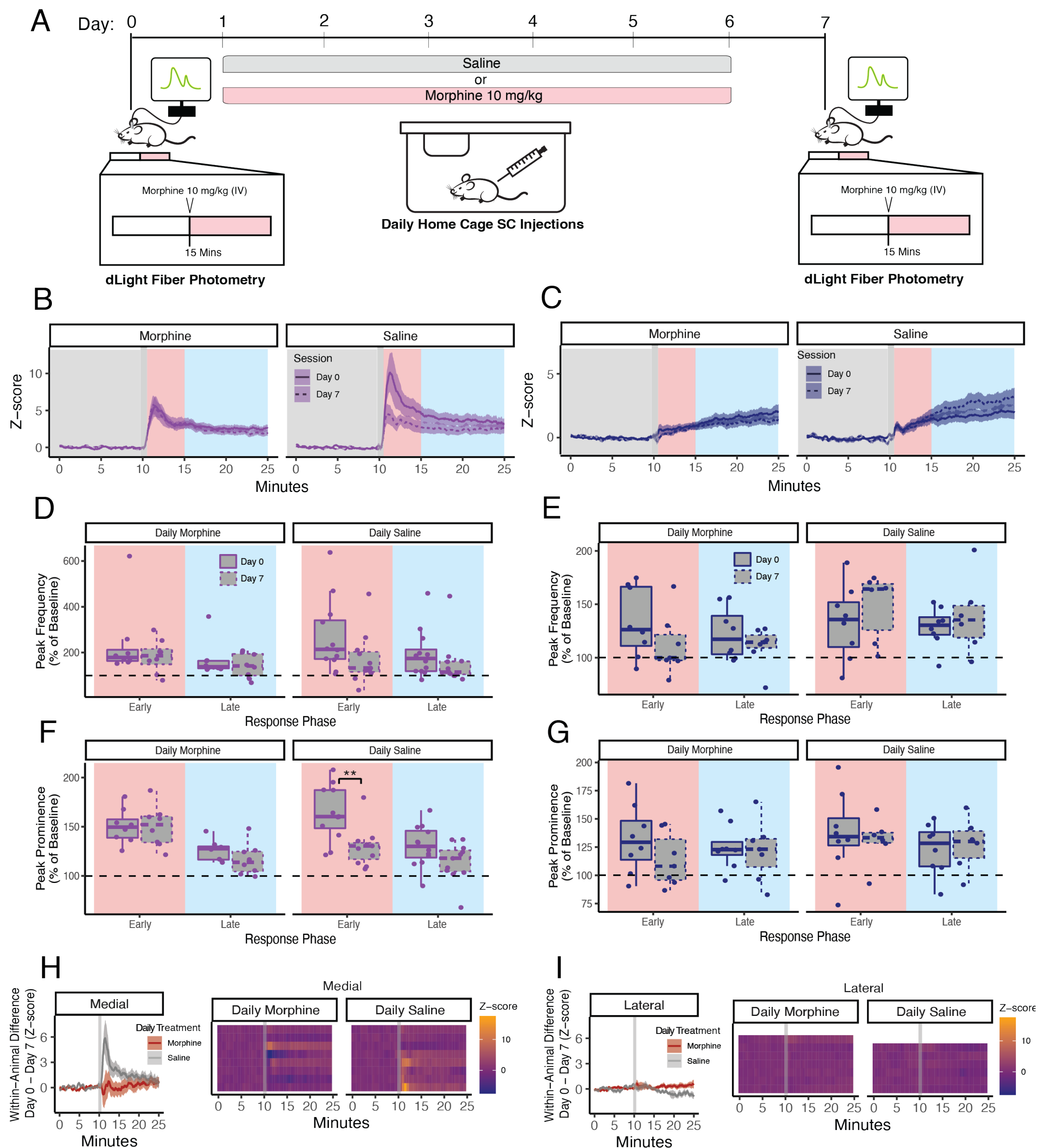

Figure 3: Recurrent morphine treatment does not shift dopamine response to IV morphine in the NAc shell. A) Timeline of longitudinal treatment paradigm. Mice received either morphine (10 mg/kg SC) or saline injection on days 1-6. On Days 0 and 7, identical fiber photometry recordings were conducted in which IV morphine (10 mg/kg) was injected 15 minutes into the baseline period. B) Average dopamine response to IV morphine injection in the medial shell of the nucleus accumbens on day 0 (solid line) and day 7 (dashed line). C) Average dopamine response to IV morphine injection in the lateral shell of the nucleus accumbens on day 0 (solid line) and day 7 (dashed line). D,E) Peak frequency in the medial (D) and lateral (E) NAc shell during early component and late component response periods after IV injection of morphine. Data is normalized to peak frequency during the pre-injection baseline period.

Figure 3 continued: F,G) Peak prominence in the medial (F) and lateral (G) NAc shell at baseline, early component and late component response periods before and after IV injection of morphine. Data is normalized to peak frequency during the pre-injection baseline period. There was a significant difference in the morphine response between day 0 and day 7 in mice treated with daily saline ($p = 0.005$, Tukey's multiple comparisons test after two-way ANOVA with repeated measures). H,I) Within-subject differences in smoothed dopamine signal between day 0 and day 7 in mice treated daily with morphine (red) or saline (gray. Shown as group averages (left) and individual subject differences (right). Each heatmap row represents individual subject differences for one animal in 30 second time bins. **$p < 0.01$.

recordings in mice treated daily with morphine (Fig 3D, E). A two-way ANOVA with repeated measures showed no significant interaction between recording day and daily treatment but there was a significant main effect of daily morphine treatment ($p = 0.41$). However, Tukey's multiple comparisons testing revealed no significant differences between individual groups or between recording days. When we analyzed the effects of chronic drug treatment on peak prominence, we found a significant effect of drug treatment ($p = 0.037$, Two-way ANOVA with repeated measures). Tukey's multiple comparisons test revealed a significant decrease in peak prominence after morphine injection in the medial shell of mice who received daily saline treatment ($p = 0.005$). This decrease was limited to the early component of the morphine response, consistent with the diminished response seen in the smoothed data (Fig. 3B) where the height of the initial transient is reduced but the lingering morphine effect (>5 minutes post-injection) remains similar to that seen on day 0.

To confirm that group averaging was not masking a within-subject effect, we performed within-subject analysis of the morphine-induced bulk fluorescence effect. For each mouse, we subtracted the standardized fluorescence from day 7 from that of day 0 across the recording period (Fig. 3F). Like the grouped analysis this revealed a diminished dopamine response to IV morphine on the seventh day in the medial shell of

mice that had received daily saline treatment peaking in the first 5 minutes post-injection. No other groups showed a strong effect of repeated morphine or saline treatment.

4.3.4 Morphine withdrawal is not reflected by a dopamine release change in the medial or lateral NAc shell.

Withdrawal from opioids in chronically treated animals is associated with increased inhibitory currents in dopamine neurons of the VTA [20, 21] and there is evidence of reduced dopamine release activity in the NAc following naloxone administration in opioid treated animals [38]. To compare the effect of naloxone-precipitated withdrawal on dopamine release in the medial and lateral shell of the NAc, we expanded the longitudinal model described above to include a naloxone-induced dopamine response before and after daily morphine treatment (Fig. 4A). Briefly, mice received two initial fiber photometry recordings to capture their exclusive response to naloxone injection (5 mg/kg, IV) before and after six days of saline treatment. These initial recordings allowed us to observe the effect of naloxone on dopamine release in morphine naïve mice as well as to determine if this response changed across the timing of our morphine treatment paradigm with daily handling and saline injection alone. We found no deviation from baseline dopamine levels in response to naloxone injection beyond the small deflection that results from the injection artifact (Fig. 4B). This was the case for recordings on day -9 and day -2.

Following these morphine naïve recordings, we performed the previously described recording paradigm to capture the response to IV morphine on day 0 and day 7 of the daily treatment schedule. On these recording days, mice were left on the recording apparatus where they received IV naloxone 30 minutes after their IV morphine injection. The goal of these recordings was to capture the dopaminergic changes that

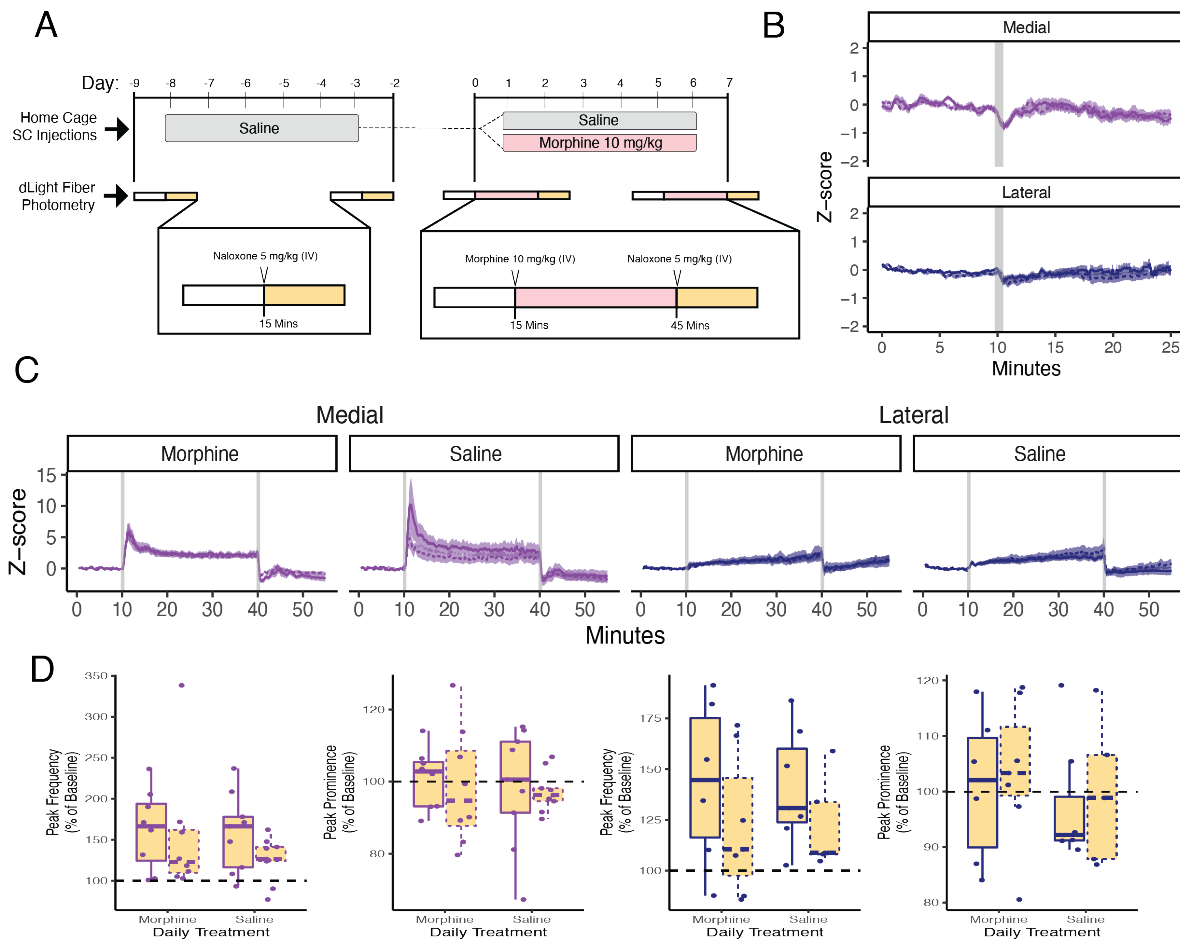

Figure 4: Morphine withdrawal is not reflected by a dopamine release change in the medial or lateral NAc shell. A) Timeline of morphine withdrawal paradigm. Morphine naïve mice received two fiber photometry (day -9 and day -2) recordings during which IV naloxone (5 mg/kg) was injected 15 minutes into the baseline period. These recordings were separated by six days on which mice received daily SC saline injections. Two days after the second naloxone recording (day 0), mice received fiber photometry recordings during which morphine was injected at 15 minutes and naloxone was injected at 45 minutes. Mice received either daily morphine or saline injection for the following six days and then a final fiber photometry recording on day 7 that matched the recording on day 0. B) Average dopamine response to IV naloxone injection in the medial and lateral shell of the NAc on day -9 (solid line) and day -2 (dashed line). C) Average dopamine response to IV morphine and subsequent naloxone injection in the medial and lateral shell of the NAc on day 0 (solid line) and day 7 (dashed line) in mice treated daily with morphine or saline. D) Peak prominence (top) and inter-peak interval (bottom) in the medial and lateral NAc shell after IV naloxone administration on day 0 (white) and day 7 (gray). E) Within-subject differences in smoothed dopamine signal between day 0 and day 7 in mice treated daily with morphine (red) or saline (gray).

occur when morphine signaling is blocked by naloxone in mice after acute morphine exposure (day 0) or repeated morphine exposure (day 7). Following these IV naloxone

injections, we observed a stark decrease in dopamine signal. This decrease was present in both the medial and the lateral shell, confirming that the slow rise in dopamine we observed in the lateral shell after morphine injection is an opioid-driven signal increase, despite being much more subtle than the increase observed in medial shell. While naloxone starkly reverses the elevated dopamine signal that follows IV morphine injection, we did not find any differences in this naloxone-precipitated decrease between day 0 and day 7 in mice that were treated daily with morphine or saline (Fig. 4C). Peak analysis of spontaneous dopamine transients following naloxone injection also showed no significant differences in peak frequency of prominence between day 0 and day 7 (Fig. 4D).

4.4 Discussion

4.4.1 Dopamine release is distinctly altered by morphine injection in the medial and lateral shell of the NAc

Here we examined dopamine release dynamics in the medial and lateral sub compartments of the shell of the nucleus accumbens in response to intravenous morphine and morphine withdrawal. Our data align with the previous understanding that the medial shell is a hotspot for opioid-induced dopamine release. In the medial shell we observed significant dopamine changes in the minutes following morphine injection exemplified by bulk dopamine rise and increased frequency and amplitude of spontaneous events. We saw a much more subdued response to IV morphine in the lateral shell of the NAc. This overall dopamine shift was also reflected in the frequency of individual dopamine release events which were significantly increased in the medial shell by morphine injection but unaffected in the lateral shell. Interestingly, the amplitude of individual release events was

significantly increased in the lateral shell despite no change in frequency and minimal bulk dopamine shift. It was fascinating to see a higher baseline frequency of spontaneous events in the lateral shell than the medial considering the finding that lateral shell projecting dopamine neurons fire more slowly than those which project to the medial shell [44]. This perhaps presents another clear example of why extracellular dopamine levels cannot be used to infer presynaptic firing activity [58].

While the medial shell has long been implicated as a critical region for acute opioid reward, few studies have appreciated this anatomical specificity. One group stands out as a leader in this regard and has published copious work comparing the roles of the NAc core and medial shell in opioid-induced dopamine release and conditioned behaviors [47, 61-66]. Notably absent from this oeuvre is a similar characterization of the NAc lateral shell. This lack of observation of the lateral mesolimbic pathway in the opioid literature is salient given the dense dopaminergic projection from the VTA to this region that, to our knowledge, has not been ruled out as an opioid-sensitive population. In a key study in 2018, one group used fiber photometry to demonstrate dopamine release in the NAc after acute heroin administration [31]. They also focused their observations on the medial shell and justified this with a quantification of cFos positive neurons in the VTA after heroin exposure. It is not clear why they chose not to explore the lateral projection further, though photometry and chemogenetic experiments can be quite resource limited. However, it is worth noting that their cFos analysis did reveal a lateral population activated by heroin, albeit fewer cells than found in the medial VTA.

We felt it was important to explore the lateral projection in parallel with the medial projecting neurons based on a seminal body of work comparing these projections in the context of rewarding and aversive stimuli [45, 46, 48-50]. Of particular note is a study showing that dopamine activity in the lateral NAc is strongly potentiated by delivery of rewarding stimuli and reward cues [49]. In this paper they demonstrate opposing responses in the ventromedial and lateral NAc shell in response to rewarding and aversive stimuli. Because acute morphine is rewarding and naloxone-precipitated morphine withdrawal is aversive, we were curious to see if these two drug-induced states mapped onto these populations in a similar way. Rather than observing similar dopaminergic potentiation by morphine in both the medial and lateral shell, we only saw this strong increase of dopamine in the medial shell though the lateral shell was not unaffected entirely. While this medial dominance was expected based on previous work, it is valuable to have it clearly demonstrated within a single experiment.

4.4.2 The mechanisms of opioid tolerance in the NAc still require extensive investigation

A key intention of this study was to characterize the effects of opioid tolerance on dopamine release in the medial and lateral NAc. Seven days of daily morphine treatment did not alter the effect of a 10 mg/kg i.v. morphine injection in either of our recording sites despite the dosing schedule being sufficient to induce analgesic tolerance and morphine dependence in our hands [67]. Reduction of dopamine activity is commonly charged as a critical mechanism of morphine tolerance, but there are surprisingly few examples in the literature of the impact of chronic opioids on dopamine release. In an exciting new study, uncaging of photoactivated opioids within the VTA caused an increase in dopamine

in the dorsomedial shell of the nucleus accumbens [68], an effect that was significantly reduced by five days of morphine treatment. Our study used intravenous drug delivery instead of localized uncaging and employed a different dosing schedule and more ventral recording location. Despite these differences in strategy, it is fascinating that our results are so different. Response to systemic morphine injection has previously been examined in rat microdialysis experiments and shown to be unchanged by daily morphine treatment [38], similarly to what we observed here. Yet another group has shown a morphine tolerance related effect in electrically evoked dopamine release [39]. However, electrically evoked and drug evoked dopamine activity can't be directly compared and the animals in this study were receiving a consistent morphine dose from implanted pellets at the time of the voltammetry recordings. Recently, dLight was used to measure the effects of chronic morphine in the core of the NAc [18]. They found some interesting changes in spontaneous activity and in the response to a fentanyl injection that were dependent on the administration pattern of chronic morphine treatment. We did not include the core as a region of interest in our study, so it is possible that this site is a substrate for morphine adaptations that has previously been under-examined in studies that centered the acute opioid response. The contrast of all these data highlights the ever-present trade-offs between mechanistic specificity and human relevance in our models and emphasizes the need to approach these questions with multiple experimental strategies.

Another surprising result of this study was the reduced response to morphine in the medial shell of mice who received saline daily. It is important to note when interpreting this result that these mice were not morphine naïve on their final recording day, but rather,

had received a single previous dose of morphine in their initial recording. The ventromedial shell has an increased dopamine response when a sucrose reward is delivered that is diminished following training to associate reward delivery with a cue [49]. It is possible that a similar mechanism is at play here and the single morphine delivery is a sufficient stimulus to adjust future dopamine responses toward that of an expected reward delivery. The implication of this data would be that chronic morphine treatment blocks this associative effect such that a morphine stimulus continues to elicit a strong transient dopamine response whether or not it is expected. There is data to suggest that drugs such as cocaine differ from natural reward sources by bypassing the diminishing dopamine response to expected stimuli that is modeled by classic reward prediction error [29]. The paradigm used here was not designed with the intention to examine the role of drug expectation. However, the context and handling associated with drug delivery may serve as cues for drug expectation. This hypothesis could be tested with some technical adjustments to the current paradigm, or perhaps in a strategically designed IV self-administration model. The covert sensitization effect we observed wherein morphine treatment blocks a desensitization effect spotlights the importance of including both within-subject and between-subject controls in studies such as ours.

4.4.3 Unique challenges accompany the search for neural correlates of opioid withdrawal

As this was the first time a high temporal resolution method has been used to measure opioid withdrawal *in vivo*, we were unsure what responses to expect in these two regions. In earlier microdialysis work, a similar dosing schedule did yield a decrease in medial shell dopamine during naloxone-precipitated withdrawal in rats [38]. However, this

withdrawal experiment took place after a day of morphine abstinence, while we precipitated withdrawal 30 minutes after a final morphine dose. We chose to precipitate withdrawal with naloxone shortly after the mice received morphine so that we could time lock our responses to the onset of withdrawal without the added variability of a more gradual protracted withdrawal pattern. The downside of this strategy was that baseline dopamine activity was altered by morphine prior to the withdrawal initiation. Without a clear picture of how basal dopamine levels change over the course of repeated morphine treatment, we chose instead to examine how the shift from this morphine-adjusted baseline differed in a within-subject model where a naloxone response was measured before and after a week of morphine administration. While naloxone effectively reversed the increased dopamine tone that follows a morphine injection, this decrease was not influenced by seven days of morphine treatment. This result was surprising as we hypothesized that, due to the known role of the nucleus accumbens in aversion, there would be a measurable effect that corresponded with the aversive state of naloxone-precipitated opioid withdrawal.

Because aversive events may depress or potentiate dopamine activity depending on the probe location, we expected that withdrawal would be accompanied by a dopamine response in one or both of our recording sites. It is possible that we did not observe any such aversion-related change because the effect of naloxone on dopamine levels is a direct downstream consequence of disinhibiting presynaptic GABA neurons. We know that aversive behaviors and accompanying dopamine modulation depend on complex circuits upstream of dopamine neurons [45, 46, 48, 49]. It is possible that the opioid-

sensitive population of GABA neurons, such as those found in the RMTg, is dominant enough to occlude modulation from other afferent inputs when a large exogenous dose of opioid agonist or antagonist is given. If this were true, the aversion-related dopamine activity that accompanies drug withdrawal would be masked by a less subtle pharmacological effect.

4.4.4 Other considerations for future interrogation the relationships between opioids and dopamine release

It is possible that our findings that morphine tolerance and withdrawal do not impact the dopamine release changes that accompany morphine and naloxone injection reflect a shortcoming of detection. Because our methods are best suited to quantify relative changes in dopamine that accompany injection events, basal changes in dopamine could still be present. We measured our morphine-precipitated increase and naloxone-precipitated decrease in dLight signal relative to a standardized baseline signal. A shifted baseline would effectively shift the entire recording such that this relative difference would remain unchanged. While we also did not detect any significant differences in the baseline fluorescence of animals before and after chronic treatment, this question is better addressed with a method that estimates true dopamine concentration. In the microdialysis literature, there are mixed results regarding basal dopamine tone and whether it is reduced after chronic morphine. Evidence exists both to support [35, 36, 61] and refute [37, 38] this hypothesis when dopamine is measured in either the core [35-37] or medial shell [38, 61] of the NAc. With no consensus on this question, more rigorous study is needed that properly differentiates the effects of morphine tolerance on basal and post-opioid dopamine levels. As dopamine sensors and optical recording methods become

more sophisticated, they may have a role in interrogating this phenomenon beyond what has been possible thus far.

We must also consider the possibility that the changes in dopamine release wrought by morphine treatment cannot be detected with the spatial resolution allowed by fiber photometry. Our experiments used a 400 um probe to collect bulk fluorescence in the surrounding tissue. Dopamine neurons are a richly heterogeneous population which can be categorized in many ways beyond projection target [69]. The variety of dopamine neuron subtypes present in the midbrain constitutes its own field of study. It is entirely possible that different dopamine neuron subtypes are modulated in contradictory ways by opioid dependence. Because dopamine neurons are a mixed population, such opposing plasticity may cancel out at the population level when measuring output from many terminals.

Finally, we must not ignore how dopamine release is influenced by factors beyond the electrical activity of dopamine neurons [70]. Dopamine neuromodulation implements volume transmission in which diffusion across a large area can reach many more cells than the tightly controlled neurotransmission of glutamatergic or GABAergic synapses [71]. This can lead to multiple timescales of transmission occurring in the same local region and blur the alignment of extracellular dopamine with activity at the cell body. Local circuitry in the nucleus accumbens also plays a major role in regulating release at the level of dopamine terminals. Cholinergic interneurons in particular have distinguished themselves as local dopamine regulators in the NAc [72, 73] where they modulate release through presynaptic input on dopamine terminals. Expression levels of the dopamine

transporter, another key regulator of extracellular dopamine, can differ based on projection target [44] and are likely altered by chronic drug treatment [74] as well. These are among the factors that add layers of complexity to the interpretation of dopamine release data, though the list is non-exhaustive.

Here we build upon previous research and provide valuable insights into the anatomical specificity of opioid-induced dopamine release by exploring both medial and lateral projections simultaneously. Furthermore, our work highlights the complexities surrounding opioid tolerance and withdrawal following chronic morphine treatment. These results underscore the need for further investigation into the mechanisms underlying opioid tolerance and withdrawal, considering the intricate interplay of neural circuits and neurotransmitter systems within the NAc. As we continue to refine our understanding of dopamine modulation in opioid use, our findings emphasize the importance of employing multifaceted experimental approaches to unravel the complexities of opioid-induced neuroadaptations.

4.5 Acknowledgements

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Chapter 5 Conclusions, Caveats, and Future Directions

5.1 Conclusions at a glance

The goal of this dissertation is to present a multi-scale analysis of the topic of opioid tolerance, dependence, and abuse liability that considers molecular mechanisms, behavioral pharmacology, and addiction-relevant neural circuits. In full, this work reflects the complexity of opioid physiology and exposes the significant gaps in the existing literature, advocating for scrupulous methods selection and emphasizing the importance of longitudinal models in future attempts to overcome this dearth of knowledge.

Chapter 2 provided an in-depth examination of the historical trajectory of opioid drug development, shedding light on the discourse surrounding biased agonism as a potential avenue for the development of safer opioid therapeutics. Additionally, we introduced a novel conceptual model to reconcile contradictions in the literature, offering a nuanced understanding of how receptor modifications and downstream cellular mechanisms synergistically contribute to opioid tolerance and dependence.

To build on this conceptual framework, in chapter 3, we presented our recent study utilizing three genotypes of mice with varied arrestin-3 recruitment abilities at the *μ*-opioid receptor (MOR) in an operant self-administration paradigm. Our findings indicated that while arrestin-3 deletion does not improve opioid abuse liability, enhancing MOR recycling may confer a protective role against compulsive drug-seeking behavior. These insights advocate for a more comprehensive consideration of the signaling profile of endogenous opioids in future drug development endeavors.

In chapter 4, we used a systems neuroscience approach and presented novel results from a longitudinal fiber photometry study to investigate dopamine release dynamics in the medial and lateral nucleus accumbens (NAc) following opioid treatment. Our findings delineated distinct patterns of dopamine release in these anatomical regions, reflecting the heterogeneous effects of opioids on discrete mesolimbic circuits. Notably, chronic morphine treatment did not alter these responses, and naloxone-precipitated morphine withdrawal did not induce shifts in dopamine release activity when compared to naloxone treatment of control animals or within-subject comparison before and after chronic morphine treatment.

Together, this dissertation lays important groundwork for a new era of opioid research that properly appreciates the heterogeneity of opioid effects and emphasizes improved approaches to drug development aimed at mitigating abuse liability while optimizing therapeutic outcomes.

5.2 Re-examining opioid drug development with a balanced signaling profile

The harmful side effects of opioid drugs such as respiratory depression, tolerance, dependence, and abuse potential have limited the therapeutic utility of opioids for their entire clinical history. However, no previous attempt to develop effective pain drugs that substantially ameliorate these effects has succeeded, and the current opioid epidemic affirms that they are a greater hindrance to the field of pain management than ever. Recent attempts at new opioid development have sought to reduce these side effects by minimizing engagement of the regulatory protein arrestin-3 at the MOR, but there is

significant controversy around this approach. In Chapter 2 we discuss the ongoing effort to develop safer opioids and its relevant historical context. We propose a new model that reconciles results previously assumed to be in direct conflict to explain how different signaling profiles at the MOR contribute to opioid tolerance and dependence. Our goal is for this framework to inform the search for a new generation of lower liability opioid analgesics.

5.3 Prioritizing disease-relevant behaviors in studies of signaling bias and side-effects

Opioid drugs are potent analgesics that mimic the endogenous opioid peptides, endorphins and enkephalins, by activating the MOR. 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 MOR impacts therapeutic utility. However, it is not clear whether increased or decreased interaction of the MOR with arrestin-3 would reduce compulsive drug-seeking. In Chapter 3, we utilized three genotypes of mice with varying abilities to recruit arrestin-3 to the MOR in response to morphine in a novel longitudinal operant self-administration model. We demonstrated that arrestin-3 knockout and wild type mice have highly variable drug-seeking behavior with few genotype differences. In contrast, in mice where the MOR strongly recruits arrestin-3, drug-seeking behavior is much less varied. We created a quantitative method to define compulsivity in drug-seeking and found that mice lacking arrestin-3 were more likely to meet the criteria for compulsivity whereas mice with enhanced arrestin-3 recruitment did not develop a compulsive phenotype. Our data

suggest that opioids that engage both G protein and arrestin-3, recapitulating the endogenous signaling pattern, will reduce abuse liability.

5.3.1 Caveats and Future Directions of Chapter 3

The study detailed in Chapter 3 attempts to supplement the existing chronicle of the relationship between MOR trafficking and opioid side-effects. Few studies in this area have examined the behavioral phenotypes that relate to abuse liability of opioid drugs, an important consideration when considering the risks and benefits of human consumption. In the study, we chose a complex longitudinal model designed to recapitulate several components of human opioid use disorder (OUD). While this model is powerful given its richness of outcome variables and relevance to human disease, such a study is challenging and subject to many limitations.

A salient consideration in this study was the choice to use an oral route of administration in the self-administration sessions. This choice was partially resource based, as IV catheterization is challenging and frequently accompanied by complications that lead to increased study attrition. It was also born of a desire to maintain a consistent administration route as the animals were drinking morphine orally in the home cage *ad libitum*. However, there are obvious caveats of oral administration that underscore the popularity of IV self-administration in substance abuse research. Morphine is predominantly administered intravenously in clinical settings and oral administration introduces variability in drug absorption, metabolism, and bioavailability compared to the IV route. Furthermore, the delayed onset of action associated with oral administration may complicate the establishment of a clear association between the drug and operant

behaviors in mice. This could pose a practical obstacle in terms of training timelines. It could also increase the variability of behavioral outcomes and complicate the interpretation of study results.

The choice of morphine as the investigational drug alone warrants some scrutiny here, not only because it isn't an ideal choice for oral delivery. While morphine is a prototypical opioid, its use is mostly constrained to highly supervised clinical settings. In models of opioid abuse behaviors, opioids more commonly associated with abuse such as oral oxycodone or IV heroin may offer greater relevance and translational value. However, these drugs have distinct pharmacodynamic profiles and abuse potential and as we were concerned with the role of arrestin-3 recruitment at the MOR, it was reasonable to choose a drug, like morphine, that has been used extensively in studies of biased agonism.

While transgenic mice are a critical tool in examining how molecular mechanisms may influence behavioral outcomes, the mutant strains used in this study were not ideally suited for such an investigation. The arrestin-3 knockout mice have a complete germline knockout of the arrestin-3 protein. Global knockout animals such as these may exhibit pleiotropic effects due to the broad disruption of physiological processes and compensatory mechanisms, potentially confounding the interpretation of study results. Knock-ins of chimeric mutant proteins with substantial sequence alterations, as is the case with the RMOR mice, may introduce alterations in protein-protein interactions, signaling pathways, or other functions, leading to unintended downstream effects unrelated to the mechanism of interest. Any such off-target effects could obscure the relevant biological consequences of the targeted manipulation.

Since the creation of the arrestin-3 knockout and RMOR mouse lines, the specific phosphorylation sites required to recruit arrestin-3 to the MOR have been identified [1]. More recently, a group has created a mouse with a targeted mutation that prevents recruitment of arrestin-3 to the MOR [2] and it would be interesting to see how this mouse performs in our model. Unfortunately, a mouse with a more refined MOR mutation to enhance arrestin-3 binding does not yet exist. A mouse we do have in our possession is the Arr-3 KO/RMOR double mutant. This mouse has the potential to alleviate concern about off-target effects that stem from the large sequence substitution of the RMOR c-tail. A lack of available arrestin-3 will reverse any RMOR phenotype that results from enhanced receptor endocytosis. Our intention is to complete another round of this study that includes these double mutant mice to further support our hypothesis on the role of arrestin-3 engagement at the MOR in the resistance to opioid abuse liability.

This study employed both male and female subjects but was not sufficiently powered to conduct an analysis of sex differences and the data reported is from mixed sexes. Mounting evidence suggests meaningful disparities between male and female subjects in opioid pharmacology and substance abuse vulnerability [3-7]. Failure to account for sex as a biological variable may lead to incomplete or skewed conclusions in studies such as this one as well as reduce the applicability of the findings toward diverse populations. Increasing the sample sizes of this study to allow for a proper interrogation of sex differences is an obvious next step.

5.4 Applying optical neuroscience techniques to the pharmacological study of chronic opioid use

It is well established that dopamine neurons of the ventral tegmental area (VTA) play a critical role in reward and aversion which is altered in several disorders, including drug addiction. While the synaptic mechanisms of opioids in the VTA have been previously characterized, this work has not sufficiently accounted for the multiple dopaminergic circuits in the midbrain, and their individual impacts on opioid dependence. Previous work suggests that different midbrain pathways, such as those targeting the medial and lateral shell of the nucleus accumbens (NAc), may play opposing roles in modulating behavior. It is possible that these functionally distinct pathways also have disparate roles in establishing opioid reward, tolerance, and withdrawal in the brain. In Chapter 4 we used a week-long morphine treatment paradigm with fiber photometry recordings of dopamine release in the medial or lateral shell of the NAc at the beginning and end of the treatment paradigm. We measured dopamine release at baseline, in response to intravenous morphine injection, and in response to a subsequent naloxone injection. Strong differences were present in these two brain regions upon the acute injection of morphine, confirming and expanding on our current understanding of opioid effects in the NAc. Chronic morphine, however, did not elicit changes in these dopamine responses. This study emphasizes many questions about how drug-induced reward and aversion may be mechanistically different from reward and aversion caused by naturalistic stimuli.

5.4.1 Caveats and Future Directions of Chapter 4

The goal of our study in Chapter Four was to examine the downstream effects of opioid-induced plasticity in the VTA using a framework that appreciates the heterogeneity

of efferent dopamine projections with respect to their anatomical targets. The combination of *in vivo* optical imaging and longitudinal drug dependence models has very little precedent. Here we describe several limitations of our study below as well as discuss key follow-up experiments needed to augment this work.

First, our photometry study used all male C57Bl/6 mice. As we discussed above, this is a clear limitation in an opioid study for many reasons. A recent study of sex differences in GABAergic plasticity of the VTA in opioid treated animals further underscores their importance [8]. Additional iterations of these experiments must include female animals and consider sex as a biological variable in further analyses. A technical caveat that bears more thorough discussion is the necessity for multiple survival surgeries in our model. The viral injection and jugular catheter implantation take place four weeks apart. We do this to prevent the attrition of animals during the viral expression wait period caused by complications with the catheter. The chance of catheter failure increases the longer they are implanted. This timeline is not ideal for an opioid study. We manage post-operative pain without the use of opioids to keep the mice opioid naïve until study entry and give a full seven days of recovery time before initiating photometry recordings whenever possible. However, the effect of post-surgical pain on the endogenous opioid system cannot be ignored.

It was necessary to deliver drugs intravenously during photometry recordings due to the time course of morphine absorption. One adaptation of this study would be to use subcutaneous fentanyl in place of IV morphine. The onset of the fentanyl-induced dopamine effect is much faster and this method has been useful to other groups [9]. In

our hands the subcutaneous fentanyl effect looks very similar to that of IV morphine. The downside of subcutaneous drug delivery is the additional handling of an awake mouse during active data collection. This can cause issues identifying a common injection time point across multiple recordings. It also introduces an artifact of animal distress from the restraint and needle poke which may be reflected in the circuitry of the dopamine reward system and confound drug-induced dopamine signals.

In this study we chose a sensor of extracellular dopamine, dLight1.3b, to monitor dopamine release changes in the medial and lateral NAc. An appropriate critique of the study is that measuring calcium activity in the terminals of dopaminergic neurons, rather than extracellular dopamine, would have been more directly relevant to elucidating the mesolimbic circuits targeted by opioid use. We chose to first focus on dopamine release due to its relevance to processes downstream of the VTA that have been implicated in the neural processes of habit forming and compulsive behavior. However, we believe that this study warrants repeating using a calcium sensor such as a cre-dependent GCaMP6 injected into the VTA such that dopaminergic terminal activity can be monitored in the NAc. A comparison of dopamine neuron activity and dopamine release is critical to the thorough understanding of these circuits given the knowledge that electrical activity and dopamine release are not always directly in concert [10].

The dLight sensors are based on a D1-type dopamine receptor conjugated to a cpGFP so that the binding of dopamine to the sensor increases its fluorescent output. While these sensors have non-functional GTPase activity and therefore initiate no signaling within their expressed neurons, there are multiple potential ways that their presence could

perturb the natural mechanisms of dopamine signaling. First, expression of any sensor necessitates the introduction of a new population of binding sites into the system. It has long been postulated that these sensors have the potential to create a sink that lowers the concentration of free molecules enough to diminish their signaling capacity, though whether this is a true concern *in vivo* is not well understood. In the case of our study, this would effectively mean that dopaminergic transmission in the system is being manipulated by the presence of the sensor itself in addition to our experimental manipulations. This could obscure or even counteract the opioid effects we are attempting to quantify and is particularly relevant in a longitudinal model that assumes changes in the dopamine system are an inevitable feature of chronic morphine exposure. Careful consideration of appropriate controls are especially critical for this reason. Another caveat of the dLight sensor is its use of the existing cellular machinery for dopamine receptor trafficking and membrane placement. Whether a highly expressed sensor could interfere with exogenous dopamine signaling by coopting cellular resources or membrane space typically reserved for the native receptors has not been determined but the possibility should not be ignored.

Contrary to our hypothesis, our study did not reveal differences in dopamine release in either the medial or the lateral NAc that were influenced by chronic treatment with morphine. This was surprising given the previously discussed changes in GABAergic inhibition that occur within the VTA [11-14]. One possibility that must be considered is that the mesolimbic projections we observed in our study are part of a separate population of dopamine neurons than those subject to these homeostatic GABA changes. These

chronic opioid mechanisms have not been examined in a way that appreciates the different projection targets of the VTA. The seminal work on opioid-induced plasticity in the VTA took place with horizontal slice preparations in assorted rodent models, methods not optimized for differentiating between efferent populations. One key experiment that must be done to rectify this is to measure the effects of chronic opioid treatment or opioid withdrawal in patch clamp studies of IPSCs in VTA dopamine neurons that have been retrogradely labeled by their target location. In fact, those experiments were intended to be a key part of this thesis but were rendered infeasible by the constraints of the COVID-19 pandemic. We maintain that a study which revisits the classical patch clamp literature on opioid tolerance and withdrawal that is updated to resolve the anatomical targets of dopamine neurons is a necessary contribution to the chronic opioid literature.

Another expansion of these studies that could address the issue of dopamine target identity would be to look at dopamine release activity (or calcium activity in dopamine terminals) at projection sites other than those considered in the current work. In theory, dopamine changes that accompany opioid tolerance and withdrawal could be occurring in sites outside of the nucleus accumbens such as the prefrontal cortex or amygdala. Applying our longitudinal fiber photometry model to these other sites would be interesting but comes with its own set of technical hurdles. The dopaminergic projection to the nucleus accumbens is incredibly dense compared to VTA projections to other regions in the brain. The sparse VTA projections to non-striatal regions has posed a challenge to many researchers attempting to study the dopamine system with photometry. New and improved dopamine sensors are now available that show more promise than dLight1.3b

in detecting signals in low dopamine regions [15]. However, limitations with our study design that make it harder to extract signal from noise, discussed later in this chapter, could bring additional challenges with respect to detecting meaningful dopamine fluctuations outside the nucleus accumbens.

While expanding this analysis to other areas of the brain is an evident direction for future studies, another endeavor that doesn't suffer the same issues of low dopamine concentration would be to take a higher resolution approach to categorizing signals within the nucleus accumbens based on the medial-lateral or dorsal-ventral axes. Comparative anatomy analysis within small brain regions poses unique challenges, including variations in mapping practices, choice of atlas, and histological techniques. These factors not only introduce a degree of error to individual projects but also complicate the comparison of studies across different laboratories. Nevertheless, it is evident that significant differences exist along these anatomical gradients concerning behavioral function and the modulation of activity in response to rewarding or aversive stimuli. Closer examination of the pivotal photometry studies that informed the present work reveals that each study targeted distinct locations within the nucleus accumbens [9, 16-18]. It is likely that a more careful cataloguing of fiber locations in a study such as ours would reveal nuanced differences beyond the broad categorizations of medial or lateral shell subdivisions.

5.4.2 Challenges of using an optical method to study pharmacology

The choice to use fiber photometry in our study in Chapter 4 gave us the ability to run large cohort sizes with data collection at multiple time points in an opioid dependence model. These factors were important given our desire to assess the effects of chronic

opioid treatment using both between-subject and within-subject controls. However, this experimental design constitutes an atypical use of fiber photometry, a relatively new method as is. Here we present a detailed discussion of the limitations of fiber photometry and particularly how they pertain to studies such as this one.

Fiber photometry and the emergence of genetically encoded fluorescent sensors has revolutionized our ability to monitor *in vivo* neural activity and neurotransmission [19]. Modern genetic tools make these sensors infinitely customizable in their expression properties, allowing for isolation of specific cellular populations and circuit distinctions such as projection targets or afferent inputs. There is a trade-off found in their sub-second temporal resolution which is slow compared to electrophysiology and electrochemical methods such as fast scan cyclic voltammetry. It is also the case that fluorescent signals will be affected by both the on and off rates of sensor binding. However, given that fiber probes are only appropriate to monitor population activity, the speed of these sensors is usually sufficient. When measuring neurochemical concentrations, optical methods are also significantly faster than microdialysis and fiber probes can be inserted with less tissue displacement than is required for standard microdialysis sampling techniques. The lightweight and flexible properties of the sampling hardware make fiber photometry ideal for monitoring activity in awake behaving animals and the ease of use of a typical photometry setup means that the method requires minimal skill acquisition beyond surgery. All these reasons have led to mass adoption of fiber photometry as a favored technique for measuring the neural correlates of animal behavior.

However, several caveats must be considered. Fluorescent sensors offer a proxy measurement for target molecules such as calcium or dopamine whereby the number of sensor proteins bound determines the amount of bulk fluorescence emitted at the fiber site. This fluorescence is modulated by internal factors such as the present level of sensor expression in the targeted neurons. It is also sensitive to external factors such as the intensity of light used for excitation of the fluorophore. For these reasons, only relative and not absolute concentration can be quantified. While a major advantage of optical methods is the ability to take several recordings in the same animal at different points in time, it remains an open question whether baseline signals can truly be compared across time points. In short, fiber photometry is best suited for describing changes that occur between different states within a recording session.

Another key concern with fiber photometry is that the strategies used to maximize data quality are varied and unstandardized throughout the field. Some of this is likely due to the novelty of the method, only a decade old. It is also true that improved materials and experimental strategies have developed so rapidly, optimization has been deprioritized as researchers race to adopt the latest tools available.

A major technical hurdle in fiber photometry is the separation of true signal from noise. This is typically accomplished with multiple phases of data processing. First, the $\Delta F/F$ calculation removes fluctuations in signal that aren't related to sensor binding. This normalization technique usually relies on a predictive model that compares signals excited by two different wavelengths, a control (isosbestic) and a binding-dependent channel. The resulting values are a corrected signal that represents sensor activity

unaffected by artifacts such as movement of the fiber. This pre-processing also frequently includes correction for photobleaching of the sensor over the recording's duration. After the raw data is corrected to extract biologically relevant signal, it is common to perform event alignment to isolate a change in signal that is specifically evoked by an experimental manipulation. This means that the recording is broken up into distinct trials which are aligned to a common time-point such as stimulus delivery and then data is averaged across trials. Event alignment is a powerful way to isolate patterns of activity that precede or follow a given stimulus or behavior and is particularly well suited for many classic behavioral paradigms.

This strategy may be less appropriate if the event in question is administration of a drug, whether contingent or non-contingent. *In vivo* pharmacological manipulations deviate from stimuli such as a foot-shocks or sound cues in unique ways. First, they are constrained by several properties that effect the movement of a drug through an animal's body known as pharmacokinetics. Drug delivery to the brain is a multi-step process if the drug first must penetrate the bloodstream and then the blood-brain barrier. Even if drugs are delivered intravenously, they vary in the speed with which they travel through tissues, and in how quickly they are metabolized and excreted.

All these processes occur upstream of changes in the calcium or neurochemicals that are the target of the chosen photometric sensor. This makes it impossible to exert the tight temporal control over a pharmacological stimulus that applies to a foot shock or a beam break sensor in a behavioral chamber. The pharmacokinetic time course also means that the magnitude of the stimulus has a continuous rather than a discrete pattern

of change meaning that where many behavioral events can be described with an on/off binary, drug exposure has a ramping on period and no singular off point. Critically, multiple deliveries of a drug will additively compound the systemic dose, a value simultaneously effected by that drug's half-life and decay. This compromises the validity of treating multiple drug infusions as discrete events for the purpose of isolating evoked changes with event alignment. While performing event analysis with multiple infusions is more appropriate with some drugs [20] than others, a different systemic dose will always apply to the baseline of each event in a session.

Beyond the technical limitations discussed here, it is also important to consider the human relevance of drug-administration patterns. We now know that drug exposure schedules can profoundly alter the impacts of a chronically administered substance [9]. The brain effects of a single large bolus of a drug are also likely to differ from the effects of several smaller, repeated doses. Experimental dosing strategies should take care to model clinical or recreational drug use patterns whenever possible. This is imperative for the construct validity of these models, an ethical concern in addition to a practical one in studies of human disease.

5.5 Closing Remarks

In closing, significant work remains to understand the mechanisms of opioids in the brain. Opioid reward, tolerance, withdrawal, and abuse liability are tightly interconnected but must not be washed over as a singular phenomenon. Opioids have had a long presence through human history and have likewise been a subject of scientific study for a long time. It is therefore easy to assume that a firm grasp on their cellular and

circuit level phenomena is at hand. In reality, there is a clear and present need to revisit many foundational studies in the field using updated methods that appreciate circuit specificity and cellular heterogeneity as well as to develop creative ways to reconcile the apparent conflicts in the literature.

As we move forward with this herculean task, we must remember that the vilification of opioid drugs is out of alignment with the goal of this work for which the common adversary is suffering and preventable death. While opioid use disorder and/or substance addiction have many associated negative outcomes, it is difficult to untangle these consequences from those which result from environmental barriers to safe and dignified drug use. More nuance is needed in how we understand and discuss opioids and their many uses both inside and outside of the clinic. The scientific community is not absolved from that obligation and rather is ideally suited to advocate evidence-based solutions free from moral judgements. My hope is that this work inspires continued study of opioids that is relevant, multifaceted, and compassionate.

5.6 References

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