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

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ABSTRACT

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

INTRODUCTION

Opioids are highly effective analgesic drugs that remain essential for the treatment of severe pain. Despite their therapeutic utility, opioid use can precipitate opioid use disorder (OUD), with 2.7 million adults in the US living with OUD[1]. Despite significant efforts and billions of dollars, the development of an opioid with reduced abuse liability has been ultimately unsuccessful. This lack of success stems in part from a poor understanding of the signaling attributes responsible for mediating the neuroplasticity that underlies OUDs.

All opioids exert their analgesic actions primarily by activating the μ-opioid receptor (MOR), a G protein-coupled receptor (GPCR) [2]. Endogenous opioid peptides, endorphins and enkephalins, bind and activate MOR to promote signaling to the $G_{i/o/z}$ G protein effectors. G protein signaling from these peptide-occupied MORs is then titrated by a cascade of events that includes phosphorylation of the MOR by GPCR kinases (GRKs) at four distinct residues [3,4] and recruitment of the arrestin-3 (β-arrestin-2) effector to the phosphorylated receptor [5]. Arrestin-3 recruitment not only uncouples MOR from G protein, thereby “arresting” G protein signaling but also promotes MOR endocytosis [6,7]. Endocytosed MORs are then dephosphorylated and recycled to the plasma membrane where they can bind ligand and initiate another cycle of signal transduction [8,9]. In contrast, while activation of the MOR by opioid drugs, including morphine and all its derivatives, also promotes G protein signaling, the morphine-activated receptors only weakly engage the GRK and arrestin-3 effectors [3,10,11], with phosphorylation of the MOR limited to one of the four residues [4] unless GRKs [5] and/or arrestins [12] are highly expressed. The endogenous opioid peptides are thus “balanced”

ligands that engage both the G protein and arrestin effectors while the opioid drugs are often “biased” ligands that favor G protein signaling in many cell types.

The impact of biased versus balanced signaling on the effect/side effect profile of opioid analgesics has been interrogated for more than two decades since the original discovery that morphine does not promote MOR endocytosis [13,14]. Nevertheless, there remains little consensus on whether arrestin-3 recruitment increases or decreases the negative side effects of opioids. This controversy persists because both eliminating arrestin-3 recruitment and enhancing arrestin-3 recruitment reduce some of the side effects of morphine. For example, mice with a disruption of the arrestin-3 gene (Arr3-KO) were reported to show increased analgesia [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 where all 11 potential phosphorylation sites are mutated to alanine (MOR 11S/T-A) are also reported to show improved analgesia and reduced analgesic tolerance, but no difference in respiratory depression in response to morphine [18]. These data would suggest that removing arrestin-3 engagement improves analgesic utility. However, promoting the recruitment of arrestin-3 by replacing the MOR with a mutant receptor that is a <u>better</u> substrate for GRKs (RMOR mice, for recycling MOR), was also reported to produce enhanced analgesia [19] and reduced analgesic tolerance to morphine with no change in respiratory depression [20]. Thus, both increasing and decreasing arrestin-3 recruitment in response to morphine improves analgesia outcomes. In addition, both decreasing (Arr3-KO mice) [21] and increasing (RMOR knock-in mice) [22] arrestin-3 recruitment were reported to show increases in the potency of morphine reward in conditioned place preference (CPP) paradigms. Dependence, defined as physical and/or affective signs of distress upon the removal of drug, is another negative side effect of opioid use and key component of OUDs. Both mouse lines deficient in arrestin-3 recruitment (Arr3-KO, MOR 11S/T-A) were reported to show intact or exacerbated morphine withdrawal signs, indicating that they still develop dependence [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 with no consensus on the best therapeutic strategy for new opioid drugs.

Perhaps the most treatment-limiting liability of opioid analgesics is the development of addiction, a syndrome in humans defined by a constellation of phenotypes that include loss of control in drug-seeking behavior, craving, and relapse. We have previously reported a three-phase operant self-administration paradigm that models compulsive drug seeking in mice: escalation of drug seeking (loss of control), failure to extinguish drug seeking (craving), and reinstatement after prolonged abstinence (relapse). Using this model, we demonstrated that WT but not RMOR mice become compulsive drug seekers with time [22]. However, no one has yet assessed how eliminating arrestin-3 engagement with MOR impacts compulsive drug seeking. Since many of the side effects of opioids are improved with both the enhancement and the elimination of arrestin-3 to the MOR, the predicted answer to this question is unclear. Whether MOR engagement

with arrestin-3 prevents or exacerbates compulsive drug seeking risk is a critical question, as the answer could inform the best strategy to develop new opioid medications with reduced abuse liability. To answer this question, we utilized our compulsive drug seeking model in mice of three genotypes with variable arrestin-3 engagement in response to morphine: WT, Arr3-KO, and RMOR.

METHODS

Mice

Mice of 3 genotypes were used in this study: 1) C57Bl/6 WT (n=26, 19 male, 7 female, 8 bred in-house and 18 purchased from the Jackson Laboratory) 2) RMOR [19] (n=15, 8 male, 7 female) bred in house, congenic >30 generations on C57Bl/6 and 3) Arr3-KO (n=16, 7 male, 9 female) originally acquired from Dr. R. Lefkowitz (Duke University) [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.

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

Oral Morphine Consumption Schedule

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

Operant Oral Self-Administration Weekly Schedule

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

Extinction

Extinction began in week 18. Three 30-minute extinction sessions were conducted every day for a maximum of 12 days (Green bars, Fig 1B). Extinction sessions were identical to operant sessions except for lever presses on the active lever during the VI period did not deliver a morphine reward or the associated tone and light cues. Mice were considered extinguished when their 3-session average of active lever presses for the day was less than 20% of their active lever presses during weeks 15-17 of their operant responding or after 12 days of extinction, whichever came first. Hence, some mice had more extinction sessions than others. All lever presses during these extinction sessions as well as days to extinguish were automatically recorded (Fig. 1C). During the extinction training, mice had access to only water (no morphine) in their home cage. This stage of our paradigm is designed to model difficulty in stopping drug-seeking.

Reinstatement

Following extinction, mice were returned to their home cage with access to only water for two additional weeks with no morphine access (light purple bar, Fig 1B). Following this abstinence period mice were returned to the operant box for a single operant session. This session consisted of a 5-minute TO period identical to previous sessions. After this TO period, the light over the active lever remained on and a single non-contingent (no lever press required) morphine reward was delivered to the port together with the light and sound cues. After this single reward delivery, the light remained on over the active lever and levers could be pressed, but no additional rewards were delivered. This stage of our paradigm is designed to model both cue- and drug-induced relapse. During this session, all lever presses, all head port entries, and the latency to collect the non-contingent reward were recorded (Fig 1C).

Scoring OUD/compulsivity

A total of 16 measures from throughout the paradigm were used to create a composite OUD/compulsivity score for each mouse (see Fig 1C for the variables used in the final score). To create this composite score for variables with significantly different raw values (lever presses on the active lever outnumber those on the inactive, for example), the raw values for each mouse for each of these 16 measures were Z-scored across the population of mice that completed the study (58 mice: 26 WT, 15 RMOR, 16 Arr3-KO). A sub-score for each of the three phases (operant, extinction, reinstatement) was then created by averaging the Z scores of each behavior in that phase for each mouse thereby giving each mouse an operant, extinction, and reinstatement sub-score where each behavior in that phase is equally weighted. A final “compulsivity score” for the entire paradigm was then created by adding the operant, extinction, and reinstatement sub-score for each mouse. Categorical assignments of compulsive or not compulsive were determined using the mean and interquartile standard deviation (IQD) composite score of WT mice. The IQD is defined as the standard deviation between Q1 and Q3. Mice with a composite score of 1 IQD or more over the mean of WT mice were designated as compulsive.

Preference

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

Study Approval

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

RESULTS

Deletion of Arr3 does not alter compulsive drug-seeking.

To determine whether the degree of arrestin-3 (Arr3) recruitment to MOR modulates compulsive drug-seeking, we compared WT, RMOR, and Arr3-KO mice in a longitudinal mouse model of OUD. These three genotypes are differentiated by their arrestin-3 recruitment to MOR. In WT mice, MORs recruit arrestin-3 in response to endorphin/enkephalin activation but only weakly in response to morphine activation (Fig 1Ai,ii). In RMOR mice, the receptor recruits arrestin-3 in response to both endorphin and morphine activation [23] (Fig 1Aiii,1Aiv). In Arr3-KO mice, the MORs have no ability to recruit arrestin-3 (schematic in Fig 1Av,1Avi).

Briefly, to monitor the transition to compulsive drug-seeking and relapse as described previously [22], we combined elements of operant self-administration and a two-bottle choice test. This longitudinal paradigm consisted of three separate stages: 1) Weekly Operant 2) Extinction and 3) Reinstatement (Fig 1B). We quantified 16 behavioral measures across the paradigm (Fig. 1C). A Principal Component Analysis (PCA) of these 16 measures revealed that RMOR mice cluster tightly together while both WT and Arr3-KO behavior have high variability (Fig 1D). We hypothesized that the variability in the WT and Arr3-KO populations reflected that some mice of those genotypes had become “compulsive”. To examine this hypothesis, we calculated a composite compulsivity score for each mouse that incorporated all 16 measures (see methods for details) and reflected their behavior relative to the entire population (Fig. 1E). We then determined which mice were outliers and designated these mice as compulsive. All compulsive mice fell outside of the RMOR cluster in the PCA (Fig. 1D solid circles). Of the 26 WT mice, 10 (38.5%) were compulsive. Of the 16 Arr3-KO mice, 6 (37.5%) were compulsive. None of the 15 RMOR mice were compulsive, replicating what we have previously shown [22]. Comparison of composite compulsivity scores showed a significant genotype effect (Fig. 1E, $p=0.0008$, $F=8.220$, one way ANOVA). When comparing composite compulsive

scores between each genotype, WT and Arr3-KO mice show no significant difference in compulsivity (p=0.57) but both WT and Arr3-KO show a significant difference in compulsivity from RMOR mice (p=0.005, p=0.0009, respectively, adjusted p-values from a one-way ANOVA with Tukey's multiple comparisons) (Fig. 1E). These data indicate that preventing arrestin-3 engagement does not reduce compulsive drug-seeking and that effective arrestin-3 engagement diminishes the liability for compulsive drug-seeking.

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

We next assessed at what point in the paradigm the compulsive and non-compulsive mice diverged in behavior. Behavior during each paradigm-specific phase was assessed using a two-way ANOVA with Tukey's multiple comparisons and reported p-values are adjusted (Fig. 2A-D). During the first three weeks of operant responding, there was no significant difference in the lever-pressing behavior between WT mice that were later identified as compulsive and WT mice that were later identified as non-compulsive mice (Fig. 2A, left gray bar vs. right gray bar). Similarly, there was no significant difference between non-compulsive Arr3-KO mice and compulsive Arr3-KO mice (Fig 2A, left orange bar vs. right orange bar). Although these values were trending towards significance (p=0.06), they did not differentiate compulsive and non-compulsive mice and were therefore not included in composite compulsivity scores.

However, by the late operant phase (weeks 15-17) there was a significant difference in lever pressing behavior between compulsive and non-compulsive WT mice (Fig. 2B, ****p ≤ 0.0001), as well as between compulsive and non-compulsive Arr3-KO mice (Fig. 2B, ****p ≤ 0.0001). Compulsive Arr3-KO and WT were indistinguishable during the late operant stage (Fig 2B). During extinction, there was also a significant difference between non-compulsive and compulsive WT mice (Fig. 2C, ****p ≤ 0.0001), and between non-compulsive and compulsive Arr3-KO mice (Fig. 2C, ****p ≤ 0.0001). There was no significant difference between non-compulsive WT, Arr3-KO, and the uniformly non-compulsive RMOR mice (Fig. 2C, left gray vs left teal vs left orange bars, ****p ≤ 0.0001). During the reinstatement stage, there was also a difference in lever pressing behavior between non-compulsive and compulsive WT mice (Fig. 2D, ****p ≤ 0.0001) and between compulsive and non-compulsive Arr3-KO mice (Fig. 2D, ****p ≤ 0.0001), but no difference between compulsive WT versus compulsive Arr3-KO mice (Fig. 2D). Non-compulsive Arr3-KO mice showed more lever pressing than both non-compulsive WT mice and RMOR mice. Overall, the lever-pressing behavior of compulsive mice and non-compulsive mice are distinguishable in every phase of the paradigm, except early operant which only trended towards significance. In addition, compulsive WT and Arr3-KO are indistinguishable from one another in lever pressing behavior in the late operant, extinction, and reinstatement phases of the paradigm. Data from Fig. 2A-D is summarized in Fig. 2E comparing compulsive mice (red lines) to non-compulsive mice (gray lines) (Fig 2E). These data demonstrate that compulsive and non-compulsive mice diverged in their

lever pressing propensity after weeks of morphine consumption and that early measures do not reliably differentiate compulsive and non-compulsive mice.

Morphine consumption does not predict compulsive drug-seeking behavior.

The vast majority of the morphine consumption in our paradigm occurred during home cage drinking. Individual mice were highly variable in their weekly morphine consumption with a range of 2.09 to 11.1mgs consumed per week, on average morphine. There was no significant difference in average morphine consumption ($p= 0.09$, $F=2.41$, one way ANOVA) between WT, RMOR, and Arr3-KO mice (Fig. 3A). The large range in total morphine consumption per mouse allowed for a linear regression analysis of compulsivity score versus consumption (Fig. 3B), which showed no correlation ($p= 0.55$, $R^2= 0.0075$). There was also no significant difference in morphine consumption between compulsive and non-compulsive mice, (Fig. 3C, $p=0.74$, $t=0.34$, unpaired t-test). These data taken together indicate that consumption of morphine does not predict liability for compulsive drug-seeking behavior.

Morphine preference does not predict compulsive drug-seeking behavior.

Another aspect of OUD is the preference of opioid drugs over other sources of positive affect. We assessed whether drug seeking behavior in this paradigm was related to voluntary consumption of morphine (a drug reward) over saccharine (a naturalistic reward). We measured morphine and saccharine consumption over four hours during week 17 of our operant paradigm with home cage morphine access. There was no significant difference in preference for morphine versus saccharin in WT, RMOR and Arr3-KO mice ($p=0.36$, $F=1.03$, one way ANOVA) (Fig. 4A). Furthermore, preference for morphine did not correlate with compulsivity score (Fig. 4B, $p=0.98$, $R^2= 3.637e-006$). Additionally, there was no significant difference in morphine preference between compulsive and non-compulsive mice (Fig. 4C, $p=0.58$, $t=0.54$, unpaired t-test). These data indicate that morphine preference does not predict liability for compulsive drug-seeking behavior.

DISCUSSION

Here we show that deletion of arrestin-3 does not protect against compulsive morphine-seeking in a mouse model of OUD. Specifically, we found that ~38% of both WT and Arr3-KO mice become compulsive drug-seekers. In contrast, none of the RMOR knock-in mice, who express a MOR that strongly engages arrestin-3, transition to compulsive drug-seeking (Fig. 1D). Importantly, we also show that neither morphine consumption (Fig. 3) nor morphine preference (Fig. 4) is predictive of compulsive drug-seeking. Degree of lever pressing early in the paradigm was likewise not predictive of compulsive drug-seeking. However, lever pressing later in the paradigm, difficulty extinguishing lever pressing, and drug/cue-induced reinstatement were significantly higher in compulsive versus non-compulsive mice (Fig. 2). These data indicate that many of the behavioral assays, including simple operant responding, CPP and consumption, used to infer “addiction” in mouse models early in their drug exposure may not be predictive. This aligns with the

observation that although reward measured by CPP is enhanced in both RMOR [22] and Arr3-KO [24] mice compared to WT mice, RMOR mice do not transition to compulsive drug-seeking [22] as we reproduce here, while both Arr3-KO and WT mice do. As morphine is rewarding in WT and Arr3-KO mice [21] that develop OUD and in RMOR mice that do not, these data indicate that any future opioids should be evaluated for abuse liability and perhaps not discarded simply because they produce “reward”. Taken together, these data also indicate that G protein-biased opioid ligands, that do not engage arrestin-3 will show no improvement in abuse liability. This is a critical finding as the primary focus of drug development for the past two decades has been towards ultra-G-biased ligands. TRV-130 (Oliceridine) is such an ultra-G-biased ligand and was FDA-approved in 2020—the first new opioid in 4 decades.

In addition to abuse liability, the development of tolerance and dependence present significant limitations to the clinical utility of morphine. The RMOR mice can provide valuable insight into how we might address these undesirable effects as well. In RMOR mice, MOR signaling in response to morphine mimics that of the endogenous opioids by promoting arrestin-3 engagement followed by endocytosis and recycling—in effect converting morphine into endorphin for signaling. RMOR mice show excellent analgesic response to morphine but do not develop tolerance under conditions where both WT [19,20] and Arr3-KO [20] mice do. In addition, RMOR mice do not show either physical [19] or affective [22] dependence while both Arr3-KO [16] and MOR 11S/T-A [18] mice show dependence at a similar level or exacerbated compared to WT mice. Importantly, the pharmacokinetics of morphine—as well as any off-target effects—are unaltered in RMOR knock-in mice and RMOR and WT mice have the same number of opioid receptors [19]. In addition, the efficacy of morphine and [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO, a hydrolysis-resistant form of enkephalin) are also equivalent both for inhibition of inwardly rectifying potassium channels and for inhibition of transmitter release in the brain [25]. The differentiating characteristic of RMOR mice is that signaling bias in response to morphine has been substantially altered to be more “balanced”, as the MOR is endocytosed and recycled in response to an analgesic dose of morphine [25].

This balance is achieved because the RMOR is a better substrate for GRKs [23], resulting in increased arrestin-3 recruitment in response to morphine. The RMOR receptor is a better substrate for GRKs because it is a chimeric receptor in which 22 amino acids of the cytoplasmic tail of MOR are replaced by those from the closely related delta opioid receptor [19,23]. While creation of this receptor was done stochastically, we now know this sequence replaced the phosphorylation bar code of the MOR [3,26] 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 [27]. Due to this 22 amino acid substitution, we cannot rule out that the RMOR but not the WT MOR signals to an unidentified effector specific to DORs and that this other effector, rather than arrestin-3 recruitment, prevents tolerance, dependence, and compulsive drug-seeking. However, deletion of the DOR actually reduces tolerance to morphine [28]. If the DOR tail mediated the reduction in tolerance seen in the RMOR through another effector, deletion of the

DOR tail would result in increased tolerance, not reduced tolerance. In addition, while morphine is a better analgesic in RMOR mice compared to WT mice, methadone analgesia is unaltered, indicating that it is a change in morphine bias not a global change in signaling that underlies the RMOR morphine phenotypes of reduced tolerance, dependence, and compulsive drug-seeking.

Recapitulating "balanced" signaling with exogenous ligands is immensely challenging. Categorizing ligands as 'balanced' or 'biased' ligands is dependent on accurately defining both arrestin-3 recruitment and signaling to $G_{i/o/z}$ G protein effectors, both of which contain caveats. For example, while morphine and all its derivates are biased for G protein signaling compared to the endogenous ligands, they do have some ability to engage arrestins. This ability varies depending on the level of GRKs and arrestins. In cells with low GRK expression, efficacy for arrestin recruitment is less than 20% even at saturating ligand concentrations [29] while in cells overexpressing GRKs [30] or arrestin-3 [5], efficacy for arrestin-3 recruitment approaches ~75% compared to DAMGO. This observation has made it difficult to assign a single signaling "bias" value for morphine—though it is always more G biased than the endogenous ligands regardless of GRK/arrestin levels [31–33]. Fentanyl and its derivatives are also G biased, because even though the efficacy of fentanyl for arrestin-3 recruitment is 100% at saturating concentrations, at the EC50 concentration for G protein there is no arrestin-3 recruitment in response to fentanyl [4]. In fact, the only clinically-utilized opioid drug that approaches a signaling bias comparable to endorphins and enkephalins is methadone [34]. None of the handful of "more balanced" tool compounds have been tested *in vivo* because either they have low potency [29], poor solubility [35], or were simply abandoned in favor of "ultra G-biased" ligands.

The past two decades have seen a strong push both preclinically and clinically towards the development of ultra G-biased opioid ligands. This push follows reports that Arr3-KO mice show increased analgesia [15] and reduced tolerance [16] and respiratory depression in response to morphine compared to WT mice, indicating that biased ligands could ameliorate these key side effects. However, several recent reports have failed to reproduce these findings in Arr3-KO mice [20,36]. And clinically, Oliceridine (TRV130), a G protein biased agonist, failed to significantly reduce respiratory depression and was therefore not an improvement over other far less expensive opioids [37]. This has led to a renewed search for drug properties other than G protein bias that could be responsible for the reduced respiratory depressive effects of ultra G-biased MOR ligands such as TRV130, PZM21 and SR17018. For example, it was recently shown that these 3 compounds have lower intrinsic efficacy for G protein than morphine [38]. If receptors in respiratory centers [39] are more sensitive to changes in intrinsic efficacy than those in analgesic circuits, this observation could explain why the lower efficacy drugs cause less respiratory depression at equi-analgesic doses. However, intrinsic efficacy at G protein is only one of many caveats. New drugs come with many pharmacodynamic changes such as affinity, on and off rate, intrinsic efficacy for G proteins and arrestins, and other potential effectors, as well as differences in pharmacokinetics and engagement with

targets other than the MOR. Changes in any of these variables could contribute to the altered effect/side effect profile observed *in vivo,* making it difficult to pinpoint whether signaling bias—or any other property—is paramount.

We posit that any reassessment of the "biased is better" hypothesis should include revisiting the "balanced is better" hypothesis [40]. There are only a handful of drug-like MOR agonists that have been reported to have a balanced signaling profile comparable to the endogenous ligands and none have been tested *in vivo* except methadone. In preclinical models, methadone produces less tolerance and less dependence than morphine [34]. And though it is only rarely used as a first line analgesic in humans because of its highly variable half-life in the population, the few controlled studies that have been done show reduced tolerance to methadone in humans (see review for studies within) [41]. But of course, methadone differs in many aspects of pharmacology from morphine—not just in pharmacokinetics [42], once again hindering the ability to conclude that bias is the defining factor in its reduced side-effect profile. One additional way to examine the role of arrestin-3 recruitment in reducing opioid side effects would be to examine tolerance, dependence, and compulsive drug-seeking to methadone in the MOR 11S/T-A knock-in mouse [18], as methadone would become biased in this mouse model. This would complement the findings from the RMOR mouse where morphine is now balanced rather than biased.

To date, in silico screens for new opioid ligands have identified only G-biased compounds. This likely reflects the crystal structures used to screen for new ligands. Specifically, it was recently shown using molecular dynamic simulations that the pose(s) displayed by a methadone-occupied MOR are distinct from those for a morphine- or TRV130-occupied-receptor [43]. This indicates that using a different docking structure might identify additional balanced MOR ligands. This is not an implausible goal, as a recent natural products library screen identified a balanced, albeit low potency, MOR agonist [29], suggesting that novel chemical entities could be identified on backbones different from our existing opioids.

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

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AUTHOR CONTRIBUTIONS

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

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FIGURE CAPTIONS

Figure 1- Modeling compulsive morphine seeking in WT, RMOR, and Arr3-KO mice. A) schematic of MOR signaling in WT, RMOR, and Arr3-KO mice in response to morphine and the endogenous ligand, endorphin. Effectors include $G_{i/o/z}$ protein (G_i, circle), Arrestin-3 (Arr3, square) B) Experimental paradigm. Top bar shows weeks where slashes indicate large gaps in the timeline. Colored blocks represent oral morphine sulfate (MS) availability in the home cage where water only is blue and increasing concentrations of MS (0.3 mg/mL, 0.5 mg/mL, and 0.75 mg/mL MS) are lightest to darkest pink (middle bar). Mice had access to morphine and water five days a week and water only the two days preceding the operant lever pressing task (see methods). Bottom shows the three phases of the paradigm. Phase 1: 17 weeks of home cage drinking, with an operant session one day per week (peach bars). Phase 2: Lever pressing behavior was extinguished in up to 12 extinction sessions (green bars). Phase 3: Cue-induced reinstatement (light blue bar) of lever pressing following a 14-day drug-free period. C) Variables included in compulsivity scores for each phase of the paradigm. Top panel shows a schematic of operant box conditions during each phase. Bottom table shows behaviors from each paradigm phase that were included in the composite compulsivity score. Lever press types include active and inactive presses in both the initial 5-minute timeout (TO) period followed by the 25-minute variable interval (VI) period. D) Principal component analysis of behaviors throughout the paradigm show that RMOR (teal) mice cluster tightly while WT (gray) and Arr3-KO (orange) mice have more variable behavior. All the compulsive mice (filled grey and orange circles) fall outside the RMOR cluster. E) Final composite compulsivity scores for each mouse. Scores of compulsive mice (closed circles) were one interquartile deviation above the mean compulsivity score of WT mice. Non-compulsive mice (open circles) were defined as all mice below this threshold. All RMOR mice fall below the threshold. WT mice significantly differed from RMOR mice, but not from Arr3-KO mice in a one-way ANOVA with Tukey's multiple comparisons correction ($p=0.0052$, $p=0.57$ respectively). Arr3-KO mice were significantly different than RMOR mice ($p=0.0009$).

Figure 2- Changes in morphine seeking through time. A) Lever pressing during the early operant phase (weeks 1-3). Compulsive and non-compulsive mice do not differ in their lever pressing behavior early in the paradigm. Bar height displays total presses during the 30-minute operant session. The color gradient indicates each lever press type (lightest to darkest: active and inactive lever presses during the timeout (TO), active and inactive during the variable interval (VI). B) Lever pressing during the late operant phase (weeks 15-17). Compulsive mice show increased lever pressing compared to non-compulsive mice (****$p \leq 0.0001$). Bar height and color gradient as in A for panels B-D. C) Extinction. Compulsive mice show increased lever pressing during extinction compared to non-compulsive mice (****$p \leq 0.0001$). D) Reinstatement. Compulsive mice show an increase in lever pressing in reinstatement compared to non-compulsive mice (****$p \leq 0.0001$) (ns: $p > .05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$). All comparisons were made using a two-way ANOVA with Tukey's multiple comparisons test;

p-values shown are adjusted. E) Summary longitudinal comparison of the data depicted in A-D for compulsive (red) vs. non-compulsive (gray) mice for each genotype in early operant, (light blue), late operant (light red), extinction day 1 and last day (light green), and reinstatement (light purple).

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

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

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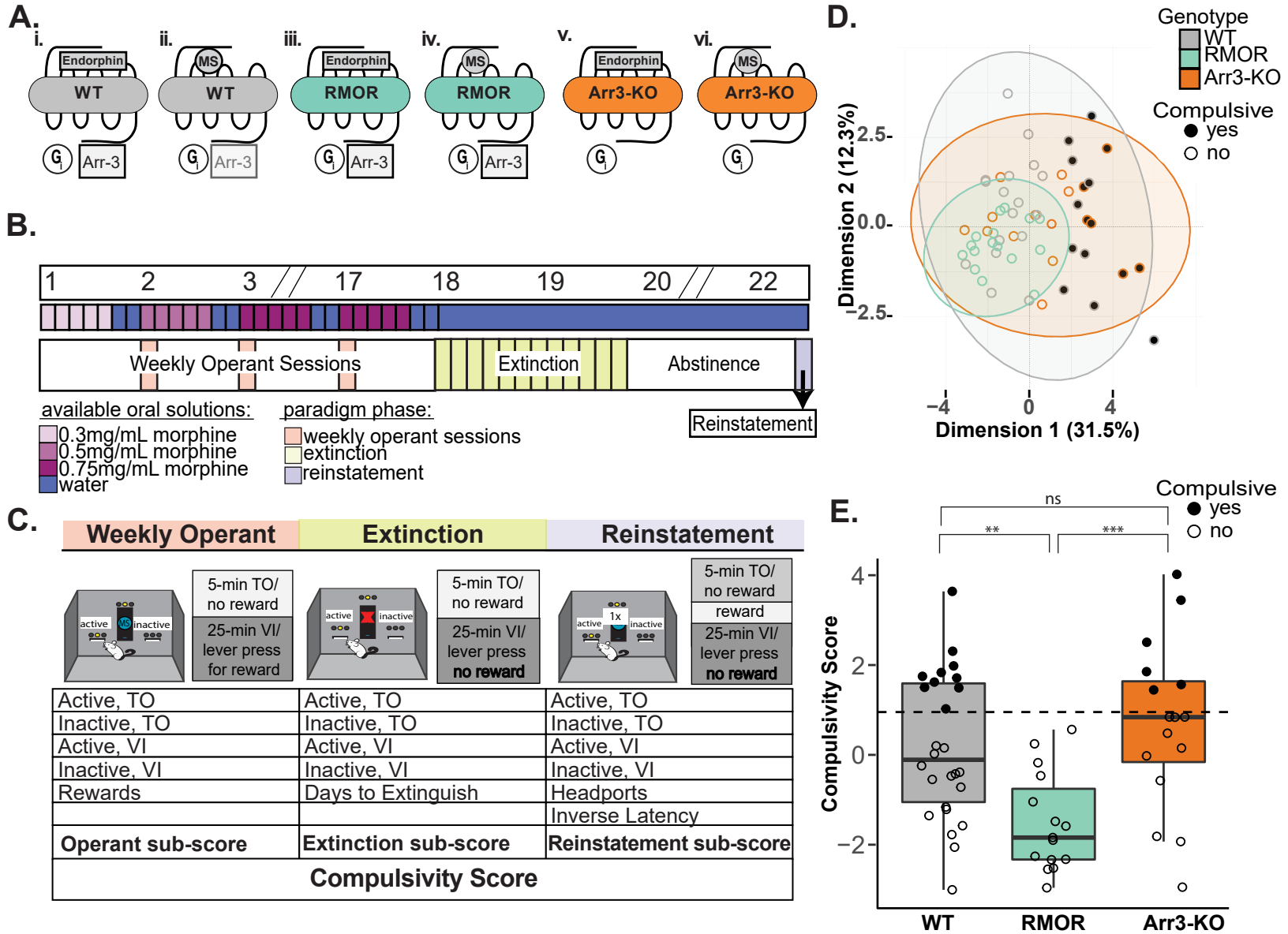
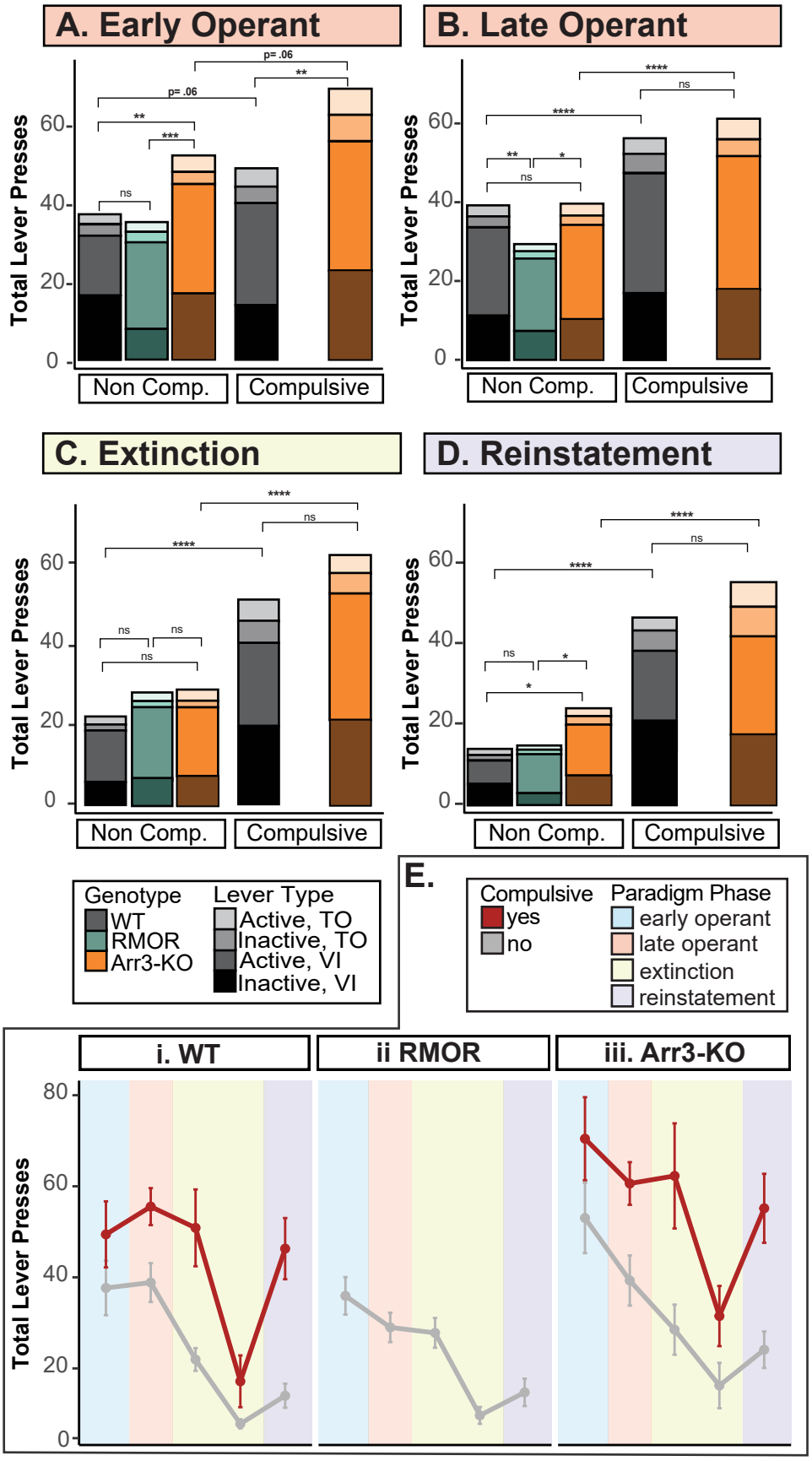

A.
i.
Endorphin
WT
Gi
Arr-3
ii.
MS
WT
Gi
Arr-3
iii.
Endorphin
RMOR
Gi
Arr-3
iv.
MS
RMOR
Gi
Arr-3
v.
Endorphin
Arr3-KO
Gi
vi.
MS
Arr3-KO
Gi
B.
1
2
3
17
18
19
20
22
Weekly Operant Sessions
Extinction
Abstinence
Reinstatement
available oral solutions:
0.3mg/mL morphine
0.5mg/mL morphine
0.75mg/mL morphine
water
paradigm phase:
weekly operant sessions
extinction
reinstatement
C.
Weekly Operant
Extinction
Reinstatement
active
MS
inactive
5-min TO/
no reward
25-min VI/
lever press
for reward
active
inactive
5-min TO/
no reward
25-min VI/
lever press
no reward
active
1x
inactive
5-min TO/
no reward
reward
25-min VI/
lever press
no reward
Active, TO
Inactive, TO
Active, VI
Inactive, VI
Rewards
Active, TO
Inactive, TO
Active, VI
Inactive, VI
Days to Extinguish
Active, TO
Inactive, TO
Active, VI
Inactive, VI
Headports
Inverse Latency
Operant sub-score
Extinction sub-score
Reinstatement sub-score
Compulsivity Score
D.
Genotype
WT
RMOR
Arr3-KO
Compulsive
yes
no
Dimension 2 (12.3%)
2.5
0.0
−2.5
−4
0
4
Dimension 1 (31.5%)
E.
Compulsive
yes
no
ns
**
***
Compulsivity Score
4
2
0
−2
WT
RMOR
Arr3-KO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B. Late Operant
C. Extinction
D. Reinstatement
Total Lever Presses
Non Comp.
Compulsive
p= .06
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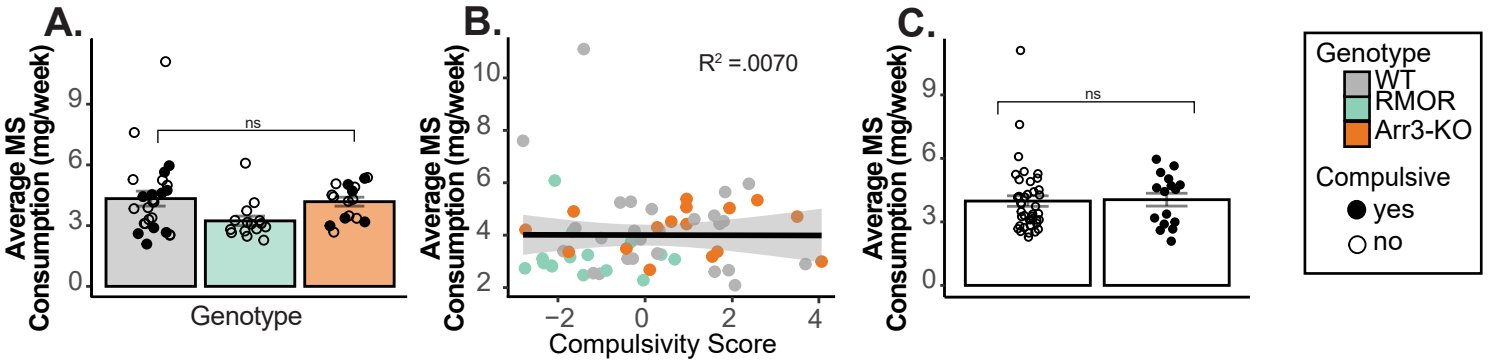
ns
Genotype
WT
RMOR
Arr3-KO
Lever Type
Active, TO
Inactive, TO
Active, VI
Inactive, VI
E.
Compulsive
yes
no
Paradigm Phase
early operant
late operant
extinction
reinstatement
i. WT
ii RMOR
iii. Arr3-KO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Average MS Consumption (mg/week)
9
6
3
0
ns
Genotype
B.
Average MS Consumption (mg/week)
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8
6
4
2
R² =.0070
−2
0
2
4
Compulsivity Score
C.
Average MS Consumption (mg/week)
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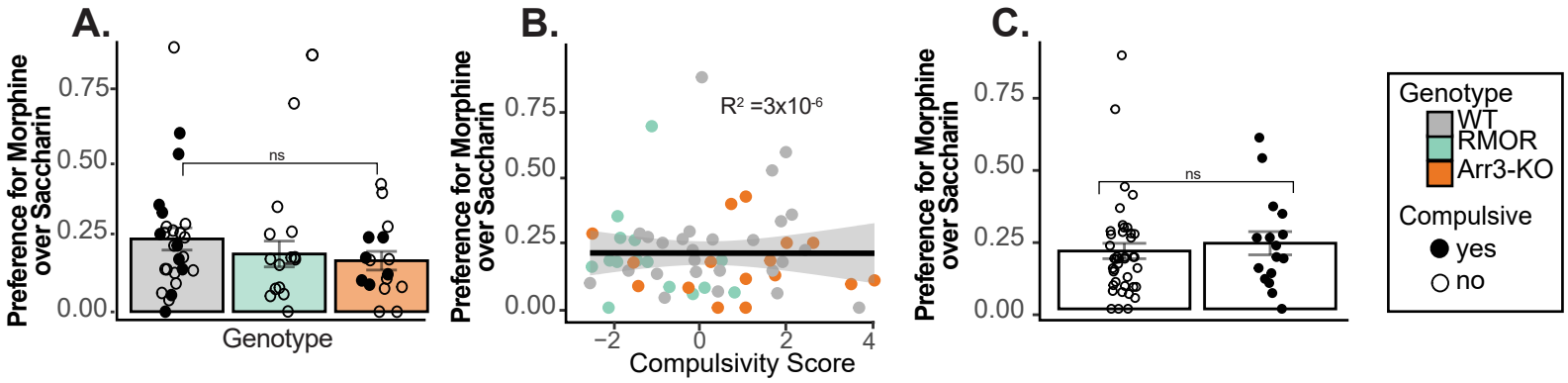
6
3
0
ns
Genotype
WT
RMOR
Arr3-KO
Compulsive
yes
no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Preference for Morphine over Saccharin
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0.50
0.25
0.00
ns
Genotype
B.
Preference for Morphine over Saccharin
0.75
0.50
0.25
0.00
R² =3x10⁻⁶
−2
0
2
4
Compulsivity Score
C.
Preference for Morphine over Saccharin
0.75
0.50
0.25
0.00
ns
Genotype
WT
RMOR
Arr3-KO
Compulsive
yes
no