Daily Intermittent Fasting in Mice Enhances Morphine-Induced Anti-Nociception while Mitigating Reward, Tolerance, and Constipation

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Author Contributions
DID conceived the initial idea for the project, collaborated on experimental and project design, performed most experiments, and analyzed most of the data. FH performed some experiments and analyzed their data. JMS trained and supervised DID and FH in the performance of the project, collaborated on experimental and project design, and analyzed some of the data. JMS and DID co-wrote the manuscript, and all authors had editorial input into the manuscript.
Abstract

The opioid epidemic has plagued the United States with high levels of abuse and poor quality of life for chronic pain patients requiring continuous use of opioids. New drug discovery efforts have been implemented to mitigate this epidemic, however, new medications are still limited by low efficacy and/or high side effect and abuse potential. Intermittent fasting (IF) has recently been shown to improve a variety of pathological states, including stroke and neuroinflammation. Numerous animal and human studies have shown the benefits of IF in these disease states, but not in pain and opioid treatment. We thus subjected male and female CD-1 mice to 18-hour fasting intervals followed by 6-hour feed periods with standard chow for 1 week. Mice which underwent this diet displayed an enhanced anti-nociceptive response to morphine both in efficacy and duration using thermal tail flick and post-operative paw incision pain models. While showing enhanced anti-nociception, IF mice also demonstrated no morphine reward and reduced tolerance and constipation. Seeking a mechanism for these improvements, we found that the mu opioid receptor (MOR) showed enhanced efficacy and reduced tolerance in the spinal cord and periaqueductal grey (PAG) respectively from IF mice using a $^{35}$S-GTPγS coupling assay. These improvements in receptor function were not due to changes in MOR protein expression. These data suggest that a daily IF diet may improve the therapeutic index of acute and chronic opioid therapies for pain patients in the clinic, providing a novel tool to improve patient therapy and reduce potential abuse.
Introduction

Opioid drugs like morphine are often the only option for moderate to severe chronic pain, but have limited efficacy in some pain types, and are limited by serious side effects, such as addiction and tolerance [3; 11; 24]. The limitations of opioid drugs, combined with an opioid abuse and overdose crisis, highlights the great medical need for new non-pharmacological approaches to improve opioid therapy. One potential approach is intermittent fasting (IF) or time restricted feeding, which is a feeding method with consistent periods in which the individual goes without food (e.g. daily 18 hour fast, 6 hour feed). From an evolutionary perspective it is plausible that our physiology evolved to operate most efficiently during long fasting periods, and that deviation from this pattern may explain many of the prevalent food-related pathologies within modern day society [19; 33]. For example, continuous insulin exposure due to standard American feeding patterns may result in tissue insulin resistance, leading to metabolic disease [7; 22; 49].

The health benefits of IF have been focused mostly on its positive effects on obesity and metabolic disorders, such as type-2 diabetes [4; 6; 29; 30]. These health benefits have also been extended to improvements in general homeostatic function and cognitive performance [14; 15; 21; 53], and in many pathological conditions including cardiovascular disease, neurological disorders, immunological disorders, and even cancer [18; 34; 35; 42; 45; 50]. Despite this literature, IF has not been tested as a strategy to improve opioid therapy, although it has been suggested for this purpose [46].

Some evidence does link fasting and dietary changes to the opioid system. For example, endogenous opioids are released within the mesolimbic dopamine system to promote food reward [10; 12; 36]. New research has also found that acute (not intermittent) fasting alters mu opioid receptor (MOR) binding, signaling, and mRNA synthesis [5; 9; 43]. However, only one study has directly studied the impact of IF on opioid function. A 16-hour daily IF protocol resulted in a circadian shift in morphine anti-nociception via hot plate in mice [52]. No other studies have addressed this question or investigated how IF might impact opioid pain therapy and side effects. Considering the many interactions of food consumption with opioid systems and the general benefits shown for IF diets, we are interested in how IF might improve opioid pharmacology. We are particularly interested in how IF might affect morphine analgesia, side effect profiles, and potential molecular mechanisms for these effects.
We thus established an 18(fast)/6 hour(feed) IF protocol with male and female CD-1 mice. After only one week of this diet, acute morphine anti-nociception was strongly enhanced in tail flick and post-operative paw incision pain models. Meanwhile, opioid-induced reward, tolerance, and constipation were all reduced or completely blocked. We further found improvements in MOR signaling in specific CNS regions that correlated with these changes, suggesting a molecular mechanism. Overall this study demonstrates a highly novel and strong benefit for IF in opioid therapy, the results of which could be quickly translated into clinical studies.

**Materials and Methods**

**Materials**

Morphine sulfate pentahydrate was obtained from the NIDA Drug Supply Program. DAMGO (#11711), naltrexone (#AAJ60013MC), naltrindole (#50-178-9293), and norBNI (#03-471-0) were obtained from Fisher Scientific (Hampton, NH). Morphine, naltrexone, naltrindole, and norBNI were prepared fresh prior to each experiment in USP saline or USP water (for i.c.v. and i.t. experiments). DAMGO was prepared as a 10 mM stock solution in water and stored at -20°C until use (single use aliquots). Saline or water vehicle controls were used in the experiments as described.

**Animals**

Male and female CD-1 mice were randomized to treatment group in age-matched controlled cohorts from 5–8 weeks of age for all behavioral experiments and were obtained from Charles River Laboratories (Wilmington, MA). CD-1 (a.k.a. ICR) mice are commonly used in opioid research as a line with a strong response to opioid drugs (e.g. [2; 27]). Mice were recovered for a minimum of 5 days after shipment before being used in experiments. Mice were housed 5 mice per cage and kept in an AAALAC-accredited vivarium at the University of Arizona under temperature control and 12-hr light/dark cycles. Control mice were provided with standard lab chow and water available ad libitum. IF mice were provided with 5 grams of food per mouse (25 grams total per cage) from 10AM to 4PM (6 hours). Notably, this feeding period was during the light cycle, the circadian inactive period for mice. Any leftover food was removed at the end of this period and repeated the next day for 1-2 weeks. Mouse body mass was monitored to be sure body mass was
maintained over the duration of the experiment. Acute 24 hour fasted mice had their food removed at 10AM with the following experiments occurring the next day at 10AM. Animals were monitored daily, including after surgical procedures, by trained veterinary staff. All experiments performed were in accordance with IACUC-approved protocols at the University of Arizona, and by the standards of the NIH Guide for the Care and Use of Laboratory Animals.

**Behavioral experiments**

Prior to any behavioral experiment or testing, the animals were brought to the testing room in their home cages for at least 1 hr for acclimation. Testing always occurred within the same approximate time of day between experiments, and environmental factors (noise, personnel, and scents) were minimized. All testing apparatus (grid boxes, etc.) were cleaned with 70% ethanol and dried between uses. The experimenter was blinded to treatment group by coding the identity of the treatment cages until after all data was collected.

**Post-surgical paw incision and mechanical allodynia**

Mechanical thresholds were determined prior to surgery using calibrated Von Frey filaments (Ugo Basile, Varese, Italy) with the up-down method and four measurements after the first response per mouse. The mice were housed in a homemade apparatus with Plexiglas walls and ceiling and a wire mesh floor (3-inch wide 4-inch long 3-inch high with 0.25-inch wire mesh). The surgery was then performed by anesthesia with ~2% isoflurane in standard air, preparation of the left plantar hind paw with iodine and 70% ethanol, and a 5-mm incision made through the skin and fascia with a no. 11 scalpel. The muscle was elevated with curved forceps leaving the origin and insertion intact, and the muscle was split lengthwise using the scalpel. The wound was then closed with 5-0 polyglycolic acid sutures. The next day, the mechanical threshold was again determined as described above. Mice were then injected with 3.2 mg/kg morphine s.c., and mechanical thresholds were determined over a 3-hour time course. No animals were excluded from these studies. This method is also reported in our previous work [27; 28].

**Tail-flick assay**
Tail-flick baselines were determined in a 52°C warm water tail-flick assay with a 10 sec cutoff time. The mice were then injected s.c. with 1-10 mg/kg of morphine, i.v. with 0.1 mg/kg morphine, i.t. with 0.1 nmol morphine, or i.c.v with 0.5 nmol morphine in saline. The procedures for i.c.v. and i.t. injection are described in our previous work [27]. For a subset of experiments, mice were injected i.p with opioid antagonists for 10 minutes prior to morphine injection and tail-flick testing. Tail-flick latencies were determined over a 2-hour time course. For tolerance studies, baseline tail flick latencies were taken, and mice were then injected with 10 mg/kg s.c. morphine with one tail flick latency measured at 30 minutes post morphine. This process was then repeated for 7 days. No animals were excluded from these studies.

**Opioid-induced constipation**

IF mice were allowed food 2 hours prior to the OIC experiment. Morphine (10 mg/kg, s.c.) was injected followed by a 6 hour fecal production time course. During this time course the mice were housed in the Von Frey boxes used to collect the paw incision data above, which had a wire mesh floor above a collection plate. The feces were counted and weighed in 1 hour bins and used to construct a cumulative plot. Morphine treated groups were then normalized to saline groups and represented as a percentage at each timepoint. AUC values from these normalized values were also quantified for a further comparison using GraphPad Prism 8.2.

**Conditioned place preference**

CPP training, baseline runs, and post-training runs were all performed in Spatial Place Preference LE 896/898 rigs. Rigs were designed to consist of two chambers with one connecting chamber. Of the two conditioned chambers, one consisted of black and grey dotted walls with a textured floor. The other chamber consisted of black and grey striped walls with a smooth floor. Chamber floors connected to a pressure sensor which transferred ongoing data to a computer running PPC WIN 2.0 software. Prior to preference training, unconditioned baselines were taken on day 0. Mice were placed in CPP chambers and allowed to roam freely for 15 minutes at ~10AM. Chambers were cleaned thoroughly with VersaClean and allowed to dry between mice. On day 1 of training mice were injected with 10 mg/kg s.c. morphine at ~10AM and placed in either stripe or dotted chambers. Half of each group paired morphine with the striped chamber and the other half
to the dotted chamber. At ~4PM mice were then given a second injection of saline which was paired to the opposite chamber. This training process was repeated for 4 days total with morning and afternoon pairings alternating each day. On day 5 mice were placed in CPP chambers and allowed to roam freely for 15 minutes at ~10AM. Raw data in the form of seconds and percentage spent in each chamber was exported from PPC WIN 2.0 as an Excel file and transferred to Prism 8.2 for further analysis.

**Western blotting and analysis**

Mouse spinal cord, PAG, and striatum protein lysates were prepared as previously reported [27] and quantified with a BCA protein quantitation assay using the manufacturer's protocol (Bio-Rad, Hercules, CA). The protein was run on precast Bolt gels (ThermoFisher, Waltham, MA) following the manufacturer's instructions. The gels were transferred to nitrocellulose membrane (Bio-Rad) using a wet transfer system (30 V, minimum of 1 h on ice). The blots were blocked with 5% nonfat dry milk in TBS and incubated with primary antibody in 5% BSA in TBS + 0.1% Tween-20(TBST) overnight rocking at 4°C. The blots were then washed three times for 5 min in TBST, incubated with secondary antibody (see below) in 5% milk in TBST for 1 hr of rocking at room temperature, washed again, and imaged with a LiCor Fc infrared imaging system (LiCor, Lincoln, NE). The blots were then stripped with 25 mM glycine-HCl and 1% SDS, pH 2.0, for 30–60 min of rocking at room temperature prior to being washed and re-exposed to primary antibody. The resulting image bands were quantified using Scion Image (based on NIH Image). All images were quantified in the linear signal range. The MOR signal was normalized to GAPDH signal for spinal cord and PAG tissues, and β-actin for striatal tissue, with both measured from the same blot as the primary target. The normalized intensities were further normalized to a vehicle control present on the same blot.

**Antibodies**

The antibodies used were: GAPDH (ThermoFisher #MA5-15738, Lot PI209504, mouse, 1:1000), β-actin (Cell Signaling #3700S, Lot 17, mouse, 1:1000), MOR (Abcam #ab134054, Lot GR180137-4, rabbit, 1:1000), secondary GαM680 (LiCor #926-68020, Lot C50721-02, goat, 1:10,000–1:20,000), and secondary GαR800 (LiCor #926-32211, Lot C50602–05, goat, 1:10,000–1:20,000).
**35S-GTPγS coupling**

35S-GTPγS (#NEG030H250UC) was obtained from PerkinElmer (Waltham, MA). Guanosine diphosphate (GDP) was obtained from Sigma Aldrich (St. Louis, MO), stored at -20°C under desiccation, made fresh for each experiment, and discarded after 60 days. Standard chemicals and buffers were purchased from Fisher Scientific with a minimum purity of 95%. Our protocol for 35S-GTPγS coupling is also reported in [38; 39; 41; 47]. Mouse tissues were dounce homogenized in homogenization buffer containing: 20 mM HEPES pH 7.0, 100 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, and centrifuged at 20,000g for 20 minutes at 4°C. The resulting pellet was resuspended in assay buffer containing: 20 mM HEPES pH 7.0, 150 mM NaCl, 2 mM MgCl2, 100 μM GDP. Concentration curves of DAMGO or vehicle were combined with 15 μg of membrane protein and 25 pM 35S-GTPγS (PerkinElmer) at a 200 μL volume using assay buffer. The reactions were incubated at RT for 2 hours. Reactions were terminated by rapid filtration through 96 well GF/B filter plates (PerkinElmer) using a 96 well format Brandel (Gaithersburg, MD) cell harvester. The plates were dried, 40 μL of Microscint PS (PerkinElmer) was added, and the data was collected using a 96 well format 6 detector MicroBeta2 scintillation counter (PerkinElmer). The resulting data was normalized to the stimulation caused by AL/Saline groups (100%) and vehicle (0%). The data was then fit with a 3 variable agonist curve, providing the potency (EC₅₀) and efficacy (E MAX), using Prism 8.2 (GraphPad). The resulting data from N = 3 independent experiments performed at least in duplicate was reported as the mean with 95% confidence intervals (CI).

**Statistical analysis**

All data was reported as the mean ± SEM or 95% CI and normalized where appropriate as described above. Paw incision and tail flick data were reported raw without maximum possible effect (MPE) or other normalization. Potency (A₅₀) values were calculated from linear regression of the tail flick dose/response data as reported in our earlier work [27]. Biological and technical replicates are described in the figure legends. Statistical comparisons were performed by Repeated Measures 2 Way ANOVA with Sidak’s (paw incision, tail flick, weight, constipation) or Tukey’s (CPP, Western) post hoc tests. Constipation AUC was compared by an Unpaired 2-Tailed t Test. In all cases, significance was defined as p < 0.05. For the 35S-GTPγS experiments, potency and efficacy values were obtained from fitted curves from N = 3 animals performed in duplicate,
reported as the mean with 95% CIs. Potency and efficacy values were considered significantly different if their 95% CIs did not overlap. All graphing and statistical analyses were performed using GraphPad Prism 8.2 (San Diego, CA). Approximately equal numbers of male and female CD-1 mice were used for each experiment. These were compared by 2 Way ANOVA with sex as a variable; no differences were observed (p > 0.05) between male and female mice, so males and females were combined together and reported as one group in each experiment.

Results

Daily intermittent fasting enhances morphine anti-nociception

Based on the human IF literature to model feasible translation, we chose a daily IF diet with an 18-hour fasting window and a 6-hour feeding window (10 AM – 4 PM, light cycle). AL and IF mice were first subjected to the post-operative paw incision pain assay after 7 days of IF; mechanical thresholds were evaluated before and after paw incision surgeries, which demonstrated no significant difference between AL and IF groups (Figure 1A-B). Upon morphine treatment (3.2 mg/kg, s.c.), IF mice displayed an enhanced duration anti-nociception vs. AL controls; this was apparent both during the fasted state at 10 AM (Figure 1A, area under the curve [AUC] increase of 66.9%) and the fed state at 4 PM (Figure 1B, AUC increase of 80.9%). This experiment suggests that acute feeding status has no impact on anti-nociception, and that enhanced anti-nociception is due to the IF diet itself.

To investigate an alternative pain type, we performed the thermal tail flick pain model and assessed thermal latencies before and after a full dose range of morphine treatment (1-10 mg/kg, s.c.). IF baselines did not differ from that of their AL counterparts, but IF mice demonstrated a significantly enhanced efficacy of morphine induced anti-nociception, similar to that of the paw incision model, that was detectable over the full dose range (Figure 1C; individual dose curves shown in Figure S1). This resulted in an approximate increase in morphine potency (A50) from 5.0 mg/kg in AL mice to 1.6 mg/kg in IF mice (Figure 1C). This finding suggests that IF enhances anti-nociception over multiple pain models and modalities (naïve vs. post-surgical; thermal vs. mechanical). We also tested whether an acute fast for 24 hours could mimic these effects, or whether a sustained IF diet was necessary. We found that a 24 hour fast had no impact on baseline
or morphine-induced responses in the tail flick assay, suggesting that a sustained IF diet is needed (Figure 1D). Lastly, we controlled for changes in weight which could impact opioid response. AL fed mice demonstrated a steady increase in body weight over the course of 7 days. Although IF mice demonstrated weight fluctuations of roughly 2-4 grams before and after eating, they also displayed a similar steady increase in body weight which was equivalent to that of the AL group at the end of each daily feed period (Figure 1E). This finding suggests that weight loss is not responsible for the enhanced morphine antinociception.

**Daily intermittent fasting blocks morphine-induced reward learning**

Opioid induced reward is a major contributing factor towards opioid addiction and dependence [23; 25]. IF has been shown to enhance brain plasticity, which might impact reward circuits and/or reward learning [46]. We thus utilized the well-established CPP assay to evaluate the effects of daily IF on morphine reward learning. AL mice demonstrated a significant preference for the morphine-paired (10 mg/kg, s.c.) chamber, which was absent in IF mice; neither group showed differences in baseline preference (Figure 2A). Food consumption is well-known to activate reward circuits, which might impact morphine reward; we thus measured morphine preference during fasted (10 AM) and fed (4 PM) states within IF mice. Both fasted and fed IF mice had no morphine preference, suggesting that blockade of morphine reward depends on sustained IF rather than acute feeding status (Figure 2B). The effects on morphine preference/reward were also consistent across the populations of each group, rather than being driven by outliers or extremes (Figure 2C). These observations thus suggest that IF mitigates or blocks morphine reward, which could be a unique translational tool to prevent pain patients from transitioning to addiction during opioid treatment. Caution must be taken however, in that CPP is an indirect measure of reward learning, and deficits in learning could potentially impact CPP without altering actual brain reward.

**Daily intermittent fasting reduces morphine-induced tolerance and constipation**

Opioid tolerance contributes to dose escalation, dependence, and addiction, while opioid-induced constipation has a strong negative impact on patient quality of life [48; 51]. We therefore investigated the effects of IF on these equally important aspects of morphine pharmacology. We induced morphine anti-nociceptive tolerance with daily 10mg/kg s.c.
Morphine injections followed by a thermal tail flick latency measurement 30 minutes after
the injection. We observed a steady decline in thermal latencies in AL mice over a 7-day
period, resulting in 94.6% tolerance; this tolerance was strongly reduced to 43.6% in IF
mice (Figure 3A). We observed no differences in saline injected controls.

To assess IF’s effects on constipation, we collected fecal matter produced from
both AL and IF mice treated with either morphine or saline over a 6-hour time course. IF
mice injected with saline produced more fecal matter over the 6 hour time course when
compared to AL mice (Figure S2A-B). To control for this difference, fecal production
collected from mice injected with morphine was normalized to that of their saline
counterparts. AL mice demonstrated a significant constipatory reduction in fecal output
with morphine treatment which was significantly reduced by the IF diet (Figure 3B-C). To
control for the impact of fasting per se on constipation, we also performed this assay with
mice acutely fasted for 24 hrs. Much like the IF mice, the 24 hr fasted mice also displayed
increased fecal production in the saline-injected controls (Figure S2C-D). However, in
contrast to the IF mice, morphine injection produced a greater constipatory effect in 24 hr
fasted mice than in AL controls (Figure 3D-E). These results show that sustained IF rather
than acute fasting is necessary to show benefits in morphine-induced constipation.
Together these experiments suggest that IF enhances the therapeutic index of morphine,
improving anti-nociception while reducing/blocking reward, tolerance, and constipation.

Altered pharmacokinetics may not explain enhancement of anti-nociception by IF

The pharmacokinetic profile of morphine has previously been shown to be altered
in leptin deficient mice [16]. It is therefore possible that the altered pattern of leptin release
in response to food intake with an IF diet could also cause pharmacokinetic differences
which might account for the enhanced morphine induced anti-nociception observed. We
thus injected equi-efficacious doses of morphine into AL and IF mice by three different
routes, intravenous (i.v., Figure 4A), intracerebroventricular (i.c.v., Figure 4B), and
intrathecal (i.t., Figure 4C). Intravenous injection circumvents absorption into the
bloodstream necessary in our previous s.c. injections, while i.c.v. and i.t. injection are
direct into the CNS, bypassing distribution through the blood-brain-barrier. In all 3 cases,
we observed an equivalent enhancement in morphine anti-nociception, very similar to the
s.c. results in Figure 1. These experiments together suggest that absorption of morphine
into the bloodstream and distribution of morphine into the CNS are not the primary
contribute factors to the observed enhanced morphine induced anti-nociception in IF mice.

*Alternate opioid receptor engagement does not explain enhancement of anti-nociception by IF*

Previous work has shown that IF can cause an increase in baseline (i.e. not in response to opioid) anti-nociception via the kappa opioid receptor (KOR) [17]. We thus used selective opioid receptor antagonists to control for the engagement of alternate opioid receptors by IF. The potent and non-selective antagonist naltrexone completely blocked morphine anti-nociception in both AL and IF mice (*Figure 4D*). In contrast, the delta opioid receptor (DOR)-selective antagonist naltrindole and the KOR-selective antagonist norBNI had no impact on either AL or IF anti-nociception (*Figure 4E-F*). Together these results strongly suggest that neither the DOR nor the KOR is activated by IF to produce morphine anti-nociception.

*Daily intermittent fasting enhances receptor efficacy and blocks receptor tolerance in spinal cord and PAG*

The majority of morphine’s effects are elicited through the MOR, which is located in specific regions of the CNS including the PAG, rostroventral medulla, striatum, and spinal cord [8]. To evaluate the molecular function of MOR in these locations in IF mice, we harvested PAG, whole brain stem, whole striatum, and whole spinal cord tissue from AL and IF mice who underwent 1 additional week of their respective diet paired with daily injections of 10mg/kg morphine or saline s.c. We then performed 35S-GTPyS coupling assays using DAMGO as a potent, selective MOR agonist. IF mice treated with saline or morphine demonstrated an enhanced efficacy when compared to their AL counterparts specifically in spinal cord (*Figure 5A, Table 1*). Morphine treated AL PAG tissue samples demonstrated a reduction of efficacy (i.e. morphine tolerance) compared to their saline counterparts, which was blocked by IF treatment (*Figure 5B, Table 1*). MOR function in striatum was largely unchanged in any group, although there was significant variability within the IF/Morphine group (*Figure 5C*). Lastly, brain stem samples demonstrated no significant difference between AL and IF groups (*Figure 5D*). Although we did observe significant differences in efficacy as described above, there were no significant differences in potency in any tissue or group (*Table 1*). These observed differences in MOR function
suggest a molecular mechanism linking IF with enhanced anti-nociception (spinal cord) and reduced anti-nociceptive tolerance (PAG).

**Daily intermittent fasting does not alter MOR protein expression in spinal cord and PAG**

We performed Western blot analysis to evaluate the role of MOR protein expression in the previously observed alterations in MOR function. Spinal cord and PAG demonstrated no observable difference in MOR expression in any treatment group (Figure 6A-B). Striatal samples demonstrated no significant differences in AL/Saline vs IF/Saline mice; interestingly, striatal samples taken from IF/Morphine mice demonstrated a significant reduction in MOR expression (Figure 6C). These results suggest that the enhanced efficacy and reduced tolerance observed in spinal cord and PAG are not due to changes in protein expression but are due to unit differences in receptor function. Our striatal results are more difficult to interpret; while no difference was observed in GTPγS function in this tissue, reduced receptor expression could have contributed to the high variability seen in this group in GTPγS. This reduction may also have contributed to the blocked morphine reward seen with IF treatment, as the striatum is a key region in the reward circuitry.

**Discussion**

Despite the modern awareness of the opioid epidemic, opioid analgesics are still commonly prescribed for the treatment of acute and chronic pain. The majority of non-opioid pain medications simply lack the efficacy required for adequate pain relief in moderate to severe pain conditions [24]. Today, roughly 19 to 43% of the United States adult population experiences chronic pain for which opioids cannot be prescribed due to insufficient efficacy and/or high side effects (i.e. poor therapeutic index)[40]. For the first time we demonstrate a non-pharmacological method of daily intermittent fasting (IF) which improved the therapeutic index of systemic morphine in mice. IF mice displayed an enhanced anti-nociceptive response in two different pain models, along with blocked reward learning and reduced tolerance and constipation. This finding suggests that IF could be used as an adjunct to opioid therapy as part of a dose reduction strategy. These findings together suggest IF may make opioid analgesic therapies more effective with reduced side effects for chronic pain patients, and may be useful in preventing the transition to addiction in chronic treatment patients. These findings are also easily
translatable, as IF is well-established in the clinical literature, with relatively high compliance, low costs, and essentially no side effects. One potential limitation to translation however is that we did not use a chronic pain model in this study, while the patients most in need of adjunct opioid therapies are chronic pain patients. The impact of IF on chronic pain anti-nociception must be studied in the future to address this gap.

Our findings also strongly distinguish IF from acute fasting and acute caloric restriction. The literature suggests that acute fasting and restriction causes a stress response in animals, deleteriously impacting opioid function and enhancing opioid reward, the opposite of what we observe [20; 43]; although some studies have shown that acute fasting and refeeding are anti-nociceptive [26]. Our control experiments further support that IF is different from acute fasting and feeding states, as a 24 hour fast and daily fasting/fed states had no impact on opioid anti-nociception, reward, or constipation (Figures 1-3). The literature and our findings thus suggest that specifically altering the timing of feeding over time is what induces long-lasting beneficial effects on opioid management of pain therapy, rather than caloric restriction, acute food-induced changes, or weight loss, which was also not impacted by IF in our study (Figure 1E). One caveat however is that our feeding time was restricted to the light cycle, which is the inactive circadian period for the mice. This restriction could induce stress or similar concerns, and could limit the impact of IF on opioid pharmacology; IF during the dark/active cycle thus must be investigated in the future, along with other variables such as different mouse strains beyond the CD-1 mice used here.

This then leaves the question of what mechanisms link IF with beneficial changes to the opioid system. Our results suggest that morphine pharmacokinetics are not altered by IF, although a direct drug measurement study would be required to make this conclusion definitely (Figure 4). The KOR and DOR also do not appear to be activated by IF (Figure 4), leaving MOR pharmacodynamics as the likely explanation. Supporting this finding, we found increased MOR efficacy in the spinal cord and blocked MOR tolerance in the PAG using 35S-GTPγS coupling (Figure 5, Table 1). The enhanced spinal cord efficacy may be responsible for the enhanced behavioral efficacy seen in paw incision and tail flick (Figure 1), while the blocked PAG tolerance may relate to the decreased behavioral tolerance observed (Figure 3A). Supporting these hypotheses, other groups have found that altering PAG signaling (e.g. ERK MAPK, microglial activation) can block
opioid tolerance [31; 32], while diabetes was shown to reduce opioid receptor coupling and thus opioid efficacy in the spinal cord [44]. Our results further suggest that unit receptor activity is altered in these tissues, since MOR protein expression is not altered in spinal cord and PAG (Figure 6A-B). Since we use a membrane preparation for our $^35$S-GTPγS studies (see Methods) which strips away all soluble components, such as kinases, cytoskeletal elements, and similar, the change in receptor activity is likely due to alterations in the receptor itself. One likely candidate would be a persistent change in receptor phosphorylation by G Protein Receptor Kinases (GRK), which cause acute desensitization and internalization of the opioid receptors [1]. Future work should investigate these potential mechanisms.

Potential mechanisms for the blockade of opioid reward learning by IF are less certain. We did observe a decrease in MOR expression in the striatum when IF was combined with chronic morphine treatment (Figure 6C). This is the inverse of what was observed with acute food restriction, where striatal MOR signaling and opioid reward were both increased, suggesting this could be the mechanism for blocked reward learning with IF [20]. However, care should be taken in interpreting this data. Our $^35$S-GTPγS coupling results showed no differences between groups in the striatum, albeit the IF/Morphine group was more variable than the rest (Figure 5C, Table 1). In addition, impacts to learning could alter CPP performance without altering actual brain reward. This mechanism will thus require more investigation. Similarly, our results with IF and constipation have no obvious mechanistic hypothesis. However, since we did observe increased fecal production in IF/Saline mice (Figure S2), IF may act to generally promote GI motility, thus counteracting the constipatory effect of morphine. Considering the large clinical burden of opioid-induced constipation [48], this mechanism merits more clinical and basic science investigation.

Although our observations have resulted in mechanistic hypotheses at the MOR functional level, we still do not know what links IF with these receptor changes. Since we are the first study to investigate the impact of IF on opioid therapy, there are also few clues from the literature. However, an earlier study did show that glucocorticoids produced from the adrenal gland were necessary for IF to alter the entrainment of circadian MOR expression [52]. Another review of the IF literature has suggested that glucocorticoids could elicit changes in Brain Derived Neurotrophic Factor (BDNF) in the brain [46]; BDNF
has been strongly linked to the regulation of opioid anti-nociception and side effects such as tolerance [32; 37]. An IF/glucocorticoid/BDNF axis could thus regulate the impact of IF on the opioid system. Another candidate is the nociceptin/orphanin-FQ system, which regulates feeding, promotes anti-nociception, and also acts as an “anti-opioid” system in the reward pathway, decreasing opioid-induced reward [13]. This and related mechanistic hypotheses must be pursued to improve our understanding of the beneficial impact of IF on opioid function and therapy and maximize our chances of successfully translating this technique to the clinic to improve opioid therapy for pain patients.

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**References**


Figure Legends

Figure 1: Daily intermittent fasting enhances morphine anti-nociception. Male and female CD-1 mice were treated with an 18/6 hour intermittent fasting (IF) protocol for 7 days, along with ad libitum (AL)-fed controls. Data reported as the mean ± SEM with the sample size of mice/group noted in each graph. Each experiment had two independent technical replicates. *, **, ***, **** = p < 0.05, 0.01, 0.001, 0.0001 vs. same time point AL group by Repeated Measures 2 Way ANOVA with Sidak’s post hoc test. A) Paw incision surgery performed on day 7 at 10 AM in the fasted state, with a 24 hour recovery and continued IF protocol. Pre- and post-surgery baselines were measured, and were not different. The mice were then injected at 10 AM on day 8 with 3.2 mg/kg, s.c. morphine with a 3 hour time course of mechanical allodynia measurement. IF enhanced morphine anti-nociception with an AUC increase of 66.9%. B) Paw incision performed as in A, except at 4 PM in the fed state. IF enhanced morphine anti-nociception with an AUC increase of 80.9%. C) Tail flick baselines were performed on day 7, which were not different between IF and AL mice. The mice were injected with 1-10 mg/kg, s.c. morphine and a 2 hour tail flick time course performed. Individual dose curves are shown in Figure S1. The area under the curve (AUC) from each dose and treatment was used to construct dose/response curves. Potency (A50) values were calculated by linear regression. A50: AL = 5.0 mg/kg; IF = 1.6 mg/kg. IF enhanced the overall potency of morphine in tail flick pain. D) Naïve mice were treated with an acute 24 hour fast, or AL controls. The tail flick assay was performed using 3.2 mg/kg, s.c. morphine. Baselines and morphine anti-nociception was not impacted by the acute fast (p > 0.05). E) Mouse body weights were recorded daily at 10 AM and 4 PM during the IF protocol (pre- and post-feeding). At 10 AM the IF groups had 2-4 g less body mass, however, body weights were the same as AL controls at the 4 PM fed period each day (p > 0.05). This suggests that the IF mice do not lose body mass during the protocol, and that the pre-feeding dip is due to the lack of bulk food mass in their GI tract after the 18 hour fasting period.

Figure 2: Daily intermittent fasting blocks morphine-induced reward. Male and female CD-1 mice were treated with the IF or AL control protocol for 7 days, with CPP conditioning beginning on day 7 (see Methods for CPP protocol). All mice received 10
mg/kg s.c. morphine as the conditioning stimulus, with saline as the counter-balanced unconditioned stimulus. Data reported as the mean ± SEM of the % time spent in the paired (morphine) chamber. Sample sizes of mice/group noted in the graphs. * = p < 0.05 vs. indicated group by Repeated Measures 2 Way ANOVA with Tukey’s post hoc test. Experiments performed in 2 independent technical replicates. A) Combined data from all mice. Baseline preference in either group was not different from the 50% mark. Morphine caused a significant preference in the AL group, which was not observed in the IF group. B) The IF data from A was split into 10 AM Fasted and 4 PM Fed groups (N = 8/each). Acute feeding status had no impact on morphine preference, with neither IF group showing a significant difference from baseline or each other (p > 0.05). C) The individual responses for each animal from each group are shown, baseline and post-conditioning. The population results suggest that the responses are consistent across each population, and are not being driven by outliers.

Figure 3: Daily intermittent fasting reduces opioid tolerance and constipation. Male and female CD-1 mice were subjected to the 7 day IF protocol or AL control, with testing beginning on day 7. Data reported as the mean ± SEM, with sample sizes of mice/group noted in the graphs. 2-4 independent technical replicates performed for each experiment. *, **, ***, **** = p < 0.05, 0.01, 0.001, 0.0001 vs. same time point AL group by Repeated Measures 2 Way ANOVA with Sidak’s post hoc test. A) Beginning on day 7, mice injected daily with 10 mg/kg morphine s.c. or saline control, with the IF or AL control protocol continuing with injections out to day 14. Tail flick latency measured 30 minutes after each daily injection and reported here. By day 7 (day 14 of total protocol), AL controls showed 94.6% tolerance, while IF mice showed 43.6% tolerance. B) On day 7, mice were injected with 10 mg/kg morphine s.c. or saline control with fecal mass in grams measured over a 6 hour time course in a cumulative plot. The morphine treatment groups were normalized to % of saline control at each time point. AUC values were also reported for each group; **** = p < 0.0001 by Unpaired 2-Tailed t Test. C) The fecal count for B reported; * = p < 0.05 by Unpaired 2-Tailed t Test. D-E) Constipation experiments performed as in B-C, except the IF group was replaced with a group of naïve mice with a 24 hr acute fast. The acute fast was followed by a 2 hr refeeding period, and then 10 mg/kg morphine s.c. and
the constipation assay as above. Acute fasting produced enhanced constipation vs. AL control, in contrast to IF findings above.

**Figure 4: Altered pharmacokinetics or alternate opioid receptor activation do not explain benefits of intermittent fasting.** Male and female CD-1 mice treated with 7 day IF protocol or AL control. On day 7, mice injected with equi-efficacious doses of morphine as noted below, with a tail flick time course. Data reported as the mean ± SEM with the sample sizes noted in the graphs. Two independent technical replicates were performed for each experiment. *, **, ***, **** = p < 0.05, 0.01, 0.001, 0.0001 vs. same time point AL group by Repeated Measures 2 Way ANOVA with Sidak’s post hoc test. A) 0.1 mg/kg morphine injected by the i.v. route. B) 0.5 nmol morphine injected by the i.c.v. route. C) 0.1 nmol morphine injected by the i.t. route. All 3 routes show equivalent enhancement of anti-nociception by IF, suggesting that IF does not alter morphine pharmacokinetics. D-F) Mice treated as above were injected i.p. with the noted dose of selective opioid receptor antagonist for 10 minutes, followed by 3.2 mg/kg morphine s.c. and a tail flick time course as above. D) Non-selective antagonist naltrexone, 2 mg/kg; naltrexone blocks all morphine anti-nociception. E) DOR-selective antagonist naltrindole, 10 mg/kg; no impact on anti-nociception. F) KOR-selective antagonist norBNI, 10 mg/kg; no impact of anti-nociception. These results suggest the MOR alone mediates morphine anti-nociception in both AL and IF mice.

**Figure 5: Daily intermittent fasting enhances MOR efficacy and reduces tolerance in spinal cord and PAG.** Male and female CD-1 mice were treated with IF or AL control for 7 days, with a further 7 days of daily morphine or saline injection with continued IF/AL protocol as in Figure 3A (14 days total). Spinal cord or brain regions were dissected and frozen, and used to perform DAMGO concentration-response curves using 35S-GTPγS coupling (see Methods). Data normalized to the max stimulation of the AL/Saline group (100%) or Vehicle control (0%) and reported as the mean ± SEM of N=3 animals/group. 3 independent technical replicates performed for each experiment. Data fit using a 3 variable non-linear regression curve. A) Spinal cord. Both IF/Saline and IF/Morphine groups had increased efficacy (see Table 1). B) PAG. Morphine treatment caused a loss of efficacy (tolerance) in the AL/Morphine group; this was not observed in the IF/Morphine group (see Table 1). C) Striatum. No differences between groups, although the IF/Morphine curve
was more variable than the others. D) Brain stem. No differences between groups.

**Figure 6: Daily intermittent fasting does not change MOR protein expression in spinal cord and PAG.** Male and female CD-1 mice treated with IF/AL and Morphine/Saline as in **Figures 3A, 5.** CNS regions analyzed for MOR protein expression by Western blot. MOR intensity normalized to GAPDH or β-actin intensity for each sample, and further normalized to the AL/Saline group, and reported as the mean ± SEM, with N = 12 mice/group. 4 independent technical replicates performed for the experiment. * = p < 0.05 vs. AL/Saline group; # = p < 0.05 vs. IF/Saline group; both by 2 Way ANOVA with Tukey’s post hoc test. Representative blots for each group shown below each graph. A) Spinal cord – no MOR expression differences (p > 0.05). B) PAG – no MOR expression differences (p > 0.05). C) Striatum – MOR expression significantly decreased in IF/Morphine group.
**A. Conditioned Place Preference**

- % Time in Paired Chamber
- BL vs. Post-Conditioning
- AL and IF groups
- N = 16

**B. CPP - Fasted vs. Fed State**

- % Time in Paired Chamber
- BL vs. Post-Conditioning
- AL, IF-Fasted, and IF-Fed groups
- N = 8-16

**C. Individual Preference Changes**

- % Time in Paired Chamber
- BL and Post-Conditioning
- AL, IF-Fast, IF-Fed, and IF-Total groups
Table 1: Potency and efficacy values for $^{35}$S-GTPγS coupling experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>AL/Saline</th>
<th>AL/Morphine</th>
<th>IF/Saline</th>
<th>IF/Morphine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>E_MAX (%)</td>
<td>EC₅₀ (nM)</td>
<td>E_MAX (%)</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>423 (273-659)</td>
<td>100 (90-111)</td>
<td>426 (257-716)</td>
<td>106 (95-119)</td>
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<tr>
<td>PAG</td>
<td>241 (188-308)</td>
<td>100 (95-105)</td>
<td>271 (165-446)</td>
<td>76* (69-84)</td>
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<tr>
<td>Striatum</td>
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<td>90 (51-187)</td>
<td>883 (531-1,500)</td>
<td>100 (85-119)</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>424 (306-590)</td>
<td>100 (92-108)</td>
<td>511 (304-871)</td>
<td>118 (104-134)</td>
</tr>
</tbody>
</table>

Mean potency (EC₅₀) and efficacy (E_MAX) values with (95% CIs) reported from curves in Figure 5. Significant differences as determined by non-overlapping CIs noted with * and highlighted in red. These values are significantly different from the same parameter from all other treatments groups in the same tissue.
Supplementary Information for:

Daily Intermittent Fasting in Mice Enhances Morphine-Induced Anti-Nociception while Mitigating Reward, Tolerance, and Constipation

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This PDF file includes:

Figures S1 to S2
Figure S1: Individual dose curves for tail flick dose/response experiment. Male and female CD-1 mice were treated with IF protocol or AL control for 7 days, with testing on day 7. On day 7, the tail flick baselines were recorded, which were not significantly different between groups (p > 0.05). The mice were then injected with 1-10 mg/kg morphine s.c. as noted in the graphs above and the tail flick response recorded over a 2 hour time course. Data reported as the mean ± SEM, with N = 10 mice/group. 2 independent technical replicates were performed for each dose. *, **, ***, **** = p < 0.05, 0.01, 0.001, 0.0001 vs. same time point AL group by 2 Way ANOVA with Sidak’s post hoc test.
Figure S2: Fecal production of saline injected controls. A-B) Male and female CD-1 mice were treated with IF protocol or AL control for 7 days, with testing on day 7. On day 7, the mice were injected with saline, and fecal mass in grams (A) or fecal number (B) was measured over a 6 hour observation period and used to construct cumulative plots. C-D) Naïve mice were treated with an acute 24 hr fast, followed by a 2 hr refeeding period and then saline injection; AL controls were also included. After injection, fecal mass (C) and number (D) was recorded as above. For all, data reported as the mean ± SEM, with N = 10-14 mice/group. 2-4 independent technical replicates were performed for each experiment. Since the IF and 24 hr fasted mice appear to produce higher levels of fecal production with saline injection, this data was used to normalize the morphine constipation data reported in the main text, as described in the main text Methods.