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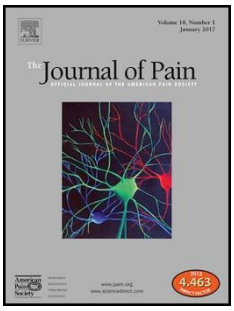

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Novel Endomorphin Analogs are More Potent and Longer Lasting Analgesics in Neuropathic, Postoperative, Inflammatory, and Visceral Pain Relative to Morphine

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Key words: Pain models; neuropathic pain; spared nerve injury; SNI; inflammatory pain; Complete Freund's Adjuvant; CFA; Postoperative pain; paw incision; visceral pain; acetic acid-induced writhing; morphine; endomorphin analogs; analgesic; glia; ZH853

Highlights

- Novel endomorphin analogs with reduced adverse effects alleviate multiple forms of pain.
- Neuropathic, inflammatory, postoperative and visceral pain are effectively relieved.
- Intrathecal injection produces more potent and longer lasting relief than morphine.
- Intravenous injection produces equal or greater potency and longer lasting relief than morphine.

Abstract

Activation of the mu-opioid receptor provides the gold standard for pain relief, but a majority of opioids used clinically have adverse effects that have contributed to an epidemic of overdose deaths. We recently characterized mu-opioid receptor selective endomorphin (EM) analogs that provide potent antinociception with reduction or absence of a number of side effects of traditionally prescribed opioids including abuse liability, respiratory depression, motor impairment, tolerance, and inflammation [91]. The current study explores the effectiveness of these EM analogs relative to morphine in four major pain models by both intrathecal and intravenous administration in male, Sprague-Dawley rats and male CD-1 mice. In the spared nerve injury (SNI) model of neuropathic pain, mechanical allodynia and mechanical hyperalgesia were assessed with von Frey and Randall-Selitto tests, respectively. In the paw incision model of postoperative pain, von Frey testing was used to assess mechanical allodynia and thermal hyperalgesia was evaluated with Hargreaves testing. In the Complete Freund's Adjuvant (CFA) model of inflammatory pain, thermal hyperalgesia was assessed by Hargreaves testing. In CD-1 mice, visceral pain was assessed with the acetic acid writhing test. In all cases, EM analogs had equal or greater potency and longer duration of action relative to morphine. The data suggest that EM analogs, particularly analog 4 (ZH853), could provide effective therapy for a diverse spectrum of pain conditions with low risk of the adverse side effects compared to currently used opioids such as morphine.

Perspective

Novel endomorphin analogs (EM analogs) show equal or greater potency and effectiveness relative to morphine in multiple pain models. Together with substantially reduced side effects, including abuse liability, the compounds show promise for addressing the critical need both for effective pain relief and reducing the opioid overdose epidemic.

Introduction

For nearly 200 years, opioid analgesics have been based on chemical structures derived from opium, which notoriously cause a host of side effects with life-threatening consequences. Recently, our lab has characterized novel endomorphin (EM) analogs that are based on the structures of endogenous EM1 and EM2 and have a strikingly reduced side effect profile compared to morphine at equiantinociceptive doses [91]. We showed an absence or reduction of abuse liability, respiratory depression, motor impairment, tolerance, and glial activation. Here, several established models of pain are used to further characterize the EM analogs in clinically relevant pain states for which they would likely be used.

Neuropathic pain affects 2 million Americans and is commonly thought to be resistant to treatment by opioids. In randomized clinical trials, fewer than half of neuropathic pain patients experience clinically meaningful pain relief with first line treatments [24,25,29]. Although efficacious, opioids are not recommended for first line treatment of neuropathic pain due to risk of adverse effects [24,25], Spared nerve injury (SNI), a model which is clinically relevant for traumatic neuropathic pain where only partial damage has occurred to a nerve, causes both mechanical allodynia and hyperalgesia [20].

Inflammatory pain states modeled in rodents with Complete Freund's Adjuvant (CFA) injections into a hind paw show localized and persistent mechanical and thermal hyperalgesia that is responsive to opioids [31,43,73]. With age, inflammation generally increases throughout the body, including conditions like arthritis [15,72]. Clinically, weak opioids have been helpful in only a subset of arthritis patients and, while strong opioids are effective, their use is limited by side effects including an increased risk of falls [50,85].

Postoperative pain is extremely common and one of the main causes of a prolonged hospital stay [1]. Extended release epidural morphine (DepoDur™) and fentanyl transdermal patches (Duragesic™) are common following surgery, but still have a high risk of adverse side effects [36,81]. Here we use the paw incision (PI) model of postoperative pain which induces mechanical allodynia and hyperalgesia, directly modeling clinical scenarios [6].

Visceral pain is one of the most common reasons that adults in the US seek medical attention for pain [84]. The acetic acid writhing test is well established [16,77] and has been used to assess morphine, endomorphin-1, and other opioids previously [4,54,75].

All pain models and methods of assessment used in this study have been used extensively to characterize analgesics [3,4,16,27,40,45,47,49,54-56,58,68,75-77,92,94]. Time course data at baseline and following either bolus or cumulative dosing with vehicle, morphine, and EM analogs were collected to determine potency and duration of action after intrathecal (i.t.) and intravenous (i.v.) administration. Visceral pain was assessed by the inhibition of writhing behavior following a subcutaneous (s.c.) dose of drug. We hypothesize that, consistent with our previous study [91], the EM analogs, particularly ZH853 (analog 4), will provide equal or greater antinociception compared to morphine in all these pain states.

Methods

Animals. Male Sprague-Dawley rats (~59-67 days old and 250-300 g at the beginning of experiments, Charles River, Wilmington, MA) and CD-1 mice (~5-7 weeks old and 25-30 g at testing, Charles River, Wilmington, MA) were group housed in a 12-h light/dark cycle (6am/6pm) in a temperature (68-72ºF) and humidity-controlled room with food and water provided *ad libitum*. All experiments were approved by the Tulane Institutional Animal Care and Use Committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used. No alternatives to in vivo techniques are available.

Drugs. EM analogs were synthesized as described previously [91] by American Peptide Company (Sunnyvale, CA).The EM analogs are cyclized, D-amino acid-containing peptides with the following structures: 1. Tyr-c-[D-Lys-Trp-Phe-Glu]-NH_2 2. Tyr-c-[D-Glu-Phe-Phe-Lys]-NH_2, 3. Tyr-c-[D-Lys-Trp-Phe-Asp]-NH_2 and 4. Tyr-c-[D-Lys-Trp-Phe-Glu]-Gly-NH_2. EM analogs 1, 3, and 4 are analogs of EM1 (Tyr-Pro-Trp-Phe-NH_2), and EM analog 2 is an analog of EM2 (Tyr-Pro-Phe-Phe-NH_2). Their high affinity, selectivity, and potent activation of the mu opioid receptor, as well as their stability, effectiveness by various routes of administration, BBB penetration, and improved, differential profile of adverse effects have been previously characterized [91]. Analog 4, with the laboratory designation ZH853, is our current lead for clinical development based on data in [91] and the present study. Morphine sulfate was supplied by NIDA. All drugs were dissolved in 20% polyethylene glycol (PEG) in sterile saline.

Drug injections for rats were given as described previously through indwelling jugular vein (i.v.) [82],[91] or intrathecal (i.t.) catheters [90],[91] which were inserted under 2.5% isoflurane anesthesia. Prior to

incision, rats received a local anesthetic (lidocaine: bupivacaine, 7mg/kg/ml:8mg/kg/ml, s.c.). Streptokinase (0.1 ml) was given to maintain patency of i.v. catheters every other day. Animals with obstructed or misplaced catheters were euthanized and excluded from the study. Before drug dosing, rats with i.t. catheters underwent lidocaine testing to determine successful placement of the catheter. After a 10 µl injection of lidocaine followed by 12µl of streptokinase, rats with properly placed catheters develop rapid but transient bilateral paralysis and recover in fewer than five minutes. Rats who did not respond or recover were immediately euthanized. I.t. drug doses were given as 6µl of drug flushed by 12µl of saline with a Hamilton syringe. I.v. injection volume was calculated by the weight of the animal and followed by 0.1 ml of saline to ensure no drug was left in the catheter. Mice received subcutaneous (s.c.) injections at the nape of the neck. Drug solutions were coded and the experimenter was blind to treatment, which was randomized in each test group.

Where possible (neuropathic and postoperative pain models), cumulative dosing was used as described previously [91] in order to minimize the number of animals necessary to generate a dose-response curve. Briefly, a first dose was given (e.g., 1.0 mg/kg), then an additive dose (0.8 mg/kg) was given to equal the cumulative dose presented in figures (1.8 mg/kg). Pilot studies established the initial doses producing ~20% effect. Doses were increased in ¼ log increments every 20 min followed 15 min later by pain assessments until maximum responses ($\geq$ 90% recovery) were attained or 4 injections were given. Vehicle-treated animals were given 4 injections to account for the highest possible fluid volume. In SNI and PI experiments, animals were given cumulative doses at 0, 20, 40, and 60 min until either >90% analgesia was achieved or all 4 doses had been administered. In our previous studies, this paradigm provided optimal approximation to results of single bolus injections. A potential artifact with cumulative dosing in the Hargreaves apparatus is that excessive urination can alter the effect of the heat stimulus on the paw and change responses. We therefore opted to give bolus doses of drug in this test to minimize this issue. In CFA experiments, therefore, a single bolus dose was given at time 0. In writhing experiments, a single bolus drug injection occurred 20 min prior to injection with acetic acid.

Pain Models. All catheters were placed in the same surgical session for SNI and 5 days prior to injection with CFA or to PI surgery. Isoflurane anesthesia (4% induction, 1.5-2.5% maintenance) was used during all surgeries and CFA injection.

Spared Nerve Injury. As previously described [20], an incision was made in the skin at the site of the trifurcation of the left sciatic nerve. The overlying muscles were retracted with blunt dissection, exposing the common peroneal, tibial, and sural nerves. The common peroneal and tibial nerves were ligated with 6-0 silk (Ethicon, Somerville, NJ) and the distal adjacent nerve (2mm) was transected. Muscle was sutured with 4-0 surgical suture (Ethicon, Somerville, NJ) and skin was closed with metal wound clips. The day of SNI surgery is referred to as day 0. Testing began 10-14 days after SNI to allow optimal sensitivity to develop.

Adjuvant-induced inflammation. Hind paws were swabbed with a sterile 70% alcohol pad. As previously described [43], CFA (100µl, s.c., Sigma, St. Louis, MO) was injected into the left hind paw using a 27-gauge needle. As an internal control, the rat's right hind paw was injected with 100µl of sterile saline. Testing started 24 hours after injection. Before testing, swelling of the left paw was confirmed with a Plethysmometer (IITC Life Science Inc., Woodland Hills, CA).

Paw Incision. As previously described [7,88], a 1cm longitudinal incision was made with a No. 10 blade through the skin and fascia on the plantar aspect of the left hindpaw beginning 0.5 cm from the end of the heel. The flexor muscle was elevated with forceps and incised longitudinally. The skin was closed with 5-0 surgical suture (Ethicon, Somerville, NJ). Behavioral testing occurred 48 hours after surgery.

Acetic Acid Writhing. Twenty minutes prior to acetic acid injection, morphine, EM analog, or vehicle were administered s.c. as a bolus injection. Acetic acid (10ml/kg of 0.6% acetic acid in sterile saline) was then administered intraperitoneally (i.p.) to induce writhing [16,77]. Each animal was immediately placed in a Plexiglas arena, video recorded for a period of 30 minutes and sacrificed after recording.

Pain Assessments. All animals were monitored for signs of axotomy or porphyrin staining which would indicate extreme stress and exclude them from behavioral testing. All tests were started in the morning. After baseline measurements, animals were randomized into drug groups by an experimenter not directly involved in the behavioral testing such that average baselines for each group were similar. Animals were

acclimated for at least 30 minutes prior to testing and sacrificed immediately after the conclusion of testing except in CFA experiments where a 24-hour time point was necessary to ensure that tissue damage had not occurred. All animals were used only once to prevent drug or testing experience from confounding the study and were drug- and test-naive when the study started.

Mechanical Hyperalgesia. To test mechanical hyperalgesia in the SNI model, a Digital Paw Pressure Randall-Selitto Instrument (IITC Life Science Inc., Woodland Hills, CA) was applied to the lateral third of the hind paw with increasing pressure until the animal withdrew or vocalized. Care was taken to avoid the hairline and toes. The device gave a maximum pressure reading in grams of force. Three measurements were taken and averaged at each time point. This method has been used previously to assess morphine analgesia [80].

Mechanical Allodynia. In the SNI and PI pain models, mechanical allodynia was assessed using nylon von Frey filaments (Stoelting, Wood Dale, IL) according to the "up-down" algorithm described by Chaplan [13]. Rats were placed on wire mesh platforms in clear cylindrical plastic enclosures 10 cm in diameter and 40 cm in height. After 15 minutes of acclimation, fibers of sequentially increasing stiffness were applied to the plantar hind paw in the sural nerve territory, pressed upward to cause a slight bend in the fiber and left in place for 5 seconds [20]. Experimenters were careful to avoid the toes and hairline of the paw. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained the next stiffest fiber in the series was applied to the same paw; if a response was obtained, a less stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after the first one causing a withdrawal response allowing the estimation of the mechanical withdrawal threshold. Sensory thresholds were estimated as described previously [13]. This assay is sufficiently sensitive to detect mechanical thresholds as low as 0.02 g, and has been used to assess the effects of opioids following SNI [27] and PI [53,60]. At baseline, all animals were tested three times on each hind foot and the averages were given to a researcher not involved in the behavioral testing who then randomized the animals into drug treatment groups.

Thermal Hyperalgesia. Withdrawal latency to heat was evaluated in CFA experiments using the IITC Plantar Analgesia Meter (IITC Life Science, Inc., Woodland Hills, CA) [38]. In this procedure, the paw

was exposed to a radiant heat source (intensity 50, cutoff 20s for inflammatory experiments and intensity 70, cutoff 15s for paw incision experiments) and latency to withdraw was recorded. A high intensity projector bulb (Osram 58-8007 8V, 50W) positioned 40mm under a glass floor was projected through a 5x10 aperture in the top of a movable case that was positioned under the hind paw. Once the rat withdrew the hind paw the heat source was turned off. Latency to withdraw was recorded and 3 tests on each side were recorded and averaged. The Hargreaves test has been used commonly to assess analgesics following both PI [46] and CFA [30,41,48].

Visceral Chemical Sensitivity. The animals' behavior was recorded for 30 minutes. Acetic acid-induced writhing behavior was identified as constrictions of the abdominal muscles causing contortions of the body and extension of the hind limbs. The video was scored by at least two different observers and the number of writhes were counted.

Statistical Analysis. Data sets were analyzed with Prism (Graphpad Software, LaJolla, CA) and are expressed as mean ± standard error of the mean (SEM). Animal numbers of 5-7 per group were used based on similar experiments in our lab and others' for appropriate statistical analysis. For all time course data, two-way repeated measures ANOVAs followed by Newman-Keuls *post hoc* testing was used to determine differences in drug or dose versus vehicle or morphine over time. Area under the curve (AUC) is calculated for each time course from minute zero through the end of the test for each animal, excluding pre-surgical baselines and the 24 h timepoint in CFA experiments. Differences in group means were determined by one-way ANOVA with Newman-Keuls *post hoc* test. To calculate dose-response (DR) curves, the percentage of maximum possible effect (%MPE) was calculated as: %MPE= [(score-baseline)/(cutoff-baseline)] x 100. Maximum possible inhibition (%MPI) in the writhing test was calculated as: %MPI= [(writhes in vehicle group-writhes in drug group)/(writhes in vehicle group)] x 100. Dose-response curves and significance of shifts in the curves were analyzed with the Prism 4-parameter nonlinear regression program. "Onset" of drug action refers to the first time point at which the mean in a drug-treated group is significantly greater than that in a vehicle-treated group and "duration" is the length of time that measurements were significantly greater than those in the vehicle-treated group. For tests involving hypersensitivity to noxious stimulation (Randall-Selitto paw pressure and Hargreaves thermal

stimulus), morphine and the EM analogs produce both a reversal of the hypersensitivity (antihyperalgesia) and antinociception (a lack of response to a pressure/heat stimulus equal or greater than that producing the baseline response). By contrast, for measures of allodynia (e.g., von Frey test) responses to drug are limited to reversal of hypersensitivity to the normally non-noxious stimulus intensity at baseline (antiallodynia). %Recovery is defined as a drug-induced return from the post-surgical level of hypersensitivity to the baseline level of response. Analysis of dose-dependent %recovery allows calculation of an index of relative potency of drugs to reverse hypersensitivity in the linear portion of the curve as 100% recovery for noxious stimuli (e.g., **Fig 1B**) and 50% recovery for non-noxious stimuli (e.g., **Fig.1F**). Expressing doses in either mass [e.g., μg/rat (i.t.), mg/kg (i.v)] or molar (μmol/kg i.v.) values has advantages for usefulness and comparison to previous studies. The data here are predominantly in the former format, except for i.v. studies where the convention used significantly alters the assessment and/or direction of relative potency, in which case the more relevant molar values are also included.

Results

Endomorphin analogs reverse mechanical hyperalgesia and allodynia after induction of chronic neuropathic pain.

As expected, SNI produced increased sensitivity (responses to a lower force stimulus) to mechanical pressure (Randall-Selitto, **Fig. 1A-C**) and von Frey filaments (**Fig. 1D-G**). The pre- and post-surgical baseline averages were not statistically different among the groups in either test. In the Randall-Selitto test, morphine and EM analogs produced antihyperalgesia (return to pre-surgical baseline) and antinociception (reduced response to pressure greater than pre-surgical baseline). The total response to drug (alleviation of pressure aversion) is shown in **Fig. 1A** and the antihyperalgesia is assessed in **Fig. 1B** by calculating 100% recovery as the return to pre-surgical baseline responses and determining the ED_{100} for paw pressure testing and ED_{50} for von Frey testing. Onset and duration of drug action were determined as described in methods. Intrathecal morphine or EM analog significantly alleviated pressure aversion relative to vehicle treatment with onset at 35 minutes. Duration of relief, however, lasted 140 minutes for morphine, 180 minutes for EM analog 2, and at least 200 minutes for EM analogs 1, 3, and 4 (solid lines, top of **Fig.1A**). In addition to differences from vehicle, EM analogs 1 and 4 alleviated pressure aversion to

a significantly greater degree than morphine (dashed lines, top of **Fig. 1A**), which encouraged us to pursue these two compounds as lead candidates. The average ED_{100} of all EM analogs showed a 78-fold leftward shift in potency relative to morphine (ED_{100} 0.0216 vs 1.66 µg/rat for morphine) (**Fig. 1B,** p=0.0017). The area under the curve was also significantly greater for EM analogs 1 and 4 relative to morphine (**Fig. 1C**). Although there are several clinical applications for i.t. administration [89], those using peripheral administration such as i.v. injection are much broader. Peptide studies often require i.t. administration to avoid destruction by enzymes in blood. However, we have demonstrated remarkable stability of the EM analogs in rat and human plasma as well as their antinociceptive activity after multiple routes of peripheral administration [91]. We therefore assessed the ability of the lead compound, EM analog 4, to reverse allodynia in the von Frey test when injected i.v. following nerve injury. EM analog 4 induced reversal of mechanical allodynia that outlasted morphine by at least 40 minutes and provided significantly greater relief than morphine for at least 2h (**Fig. 1D,** dashed lines). DR curves for morphine and EM analog 4 were nearly identical when expressed as mg/kg (**Fig. 1E**), but when calculated on a molar basis (µmol/kg), a significant, 2-fold leftward shift was observed for EM analog 4 versus morphine (ED_{50} 1.8 µmol/kg vs 5.3 µmol/kg for morphine) (**Fig. 1F**, p< 0.0001). The AUC for analog 4 was also significantly greater than that of morphine (**Fig. 1G**).

Taken together, these results indicate that, although the EM analogs are more potent when administered centrally, they are also effective when administered systemically and maintain their long-lasting effects. The fact that i.v. administration of EM analog 4 and morphine produced maximum relief of allodynia at the same mg/kg doses could also help to determine human dosing, given that opioid dosing in humans is frequently calculated as “morphine equivalents”.

Endomorphin analogs dose-dependently reverse thermal hyperalgesia caused by inflammatory pain.

The CFA model of persistent inflammatory pain was used. This model produces hypersensitivity and paw edema responses that are pronounced and stable at 1-3 days and persist for 1-2 weeks after injection [93]. We therefore tested the effectiveness of morphine and EM analogs to alleviate thermal hyperalgesia at 24 hr after CFA injection. As discussed in drug methods, bolus rather than cumulative dosing was used in this test to eliminate potential artifacts from excessive urination. As with all tests, baselines for

withdrawal latency were taken three times for each hind paw before CFA injection and 24 hr later before drug injections on test day. No significant differences were observed among the groups for any pre- or post-CFA baseline and thermal hypersensitivity was significant for all groups on test day. Initial doses were selected based on other experiments presented here and in Zadina *et al*. [91].

All drugs induced dose-dependent reversal of thermal hyperalgesia as shown in time course and DR graphs (**Fig. 2A and 2B**). Because of the large number of groups represented in this dataset, comparisons for pain relief are most easily observed as an area under the curve (AUC), which represents the total area from post-CFA baseline to 180 minutes (**Fig. 2C**). Doses of 0.1 and 0.18μg of EM analog 4 provided significantly more pain relief than 10μg of morphine and the average potency of the EM analogs was 52 times greater (p=0.0012) at 1 hour as shown in the DR curves (ED_{100} 0.08 vs 4.1 μmol/kg for morphine) (**Fig. 2D**).

Having identified EM analog 4 as the most potent and longest lasting compound, we assessed the effects of this EM analog relative to morphine on inflammatory thermal hyperalgesia after i.v. administration. Four equal doses were administered for morphine and EM analog 4 and, at the highest dose for both (10 mg/kg), the effects of EM analog 4 (onset 15 min, duration 225 min) outlasted those of morphine (onset 15 min, duration 105 min) (**Fig. 3A**). Although morphine at 10 mg/kg produced a slightly faster maximal response, the DR curves (**Fig. 3B)**, indicate a loss of effect at 2 and 3 hours for morphine while EM analog 4 remained unchanged over this time course, indicating a steady, long-lasting effect. DR curves at 1 hour were not significantly shifted on a mg/kg basis (p= 0.1871) nor were they shifted on a μmol/kg basis (**Fig. 3C**). As shown in AUC graphs (**Fig. 3D**), overall relief was not different between each drug at matching doses (p=0.2476).

Endomorphin analogs reverse mechanical allodynia and thermal hyperalgesia following induction of postoperative pain.

Forty-eight hours after paw incision surgery, testing began with a pre-drug baseline to confirm the development of mechanical allodynia and thermal hyperalgesia. Pre- and post-surgical von Frey and Hargreaves scores were similar for all drug treatment groups. As in neuropathic pain experiments, cumulative dosing was used.

We first performed experiments with i.t. administration for comparison to other peptide studies [35,37]. Based on experiments performed here and in [91], EM analogs 1 and 4 were the lead compounds at the time of testing. Von Frey testing confirmed the reversal of mechanical allodynia (significantly higher than that of vehicle controls, solid lines at top of **Fig**. **4A**) by morphine (onset 35 min, duration 120 min), EM analog 1 (onset 35 min, duration at least 200 min), and EM analog 4 (onset 15 min, duration at least 220 min). The EM analogs also showed significantly greater antiallodynia than morphine (dashed lines, top of **Fig. 4A**). The DR curve (**Fig. 4B**) showed a 20-fold leftward shift of the ED_{50} of the EM analogs relative to that of morphine (ED_{50} 0.02 vs 0.43 μg/rat for morphine) ($p < 0.0001$). When comparing this to the 78-fold shift of the EM analogs against morphine in neuropathic pain, it was noted that the DR curves of the EM analogs are in approximately the same range in both tests, indicating that they are equally potent in alleviating both types of pain. Morphine, by contrast, seems to be much less effective against neuropathic pain compared to postoperative pain (ED_{50} 5.3 vs 0.43 μg/rat, respectively). The AUC for EM analogs 1 and 4 were significantly greater than morphine (**Fig. 4C**) ($p< 0.0001$).

The Hargreaves test was used to assess thermal hyperalgesia. To use cumulative dosing while limiting the effects of urine on paw responses described above, the apparatus was cleaned between each injection. I.t. morphine (onset 55 min, duration 60 min), EM analog 1 (onset 35 min, duration 100 min), and EM analog 4 (onset 35 min, duration at least 160 min) all provided relief from noxious heat aversion (significantly greater than vehicle; $p<0.05$ or greater), solid top lines, **Fig. 4D**). Both EM analogs provided longer lasting relief and analog 4 showed significantly greater relief than morphine at the last two time points (dashed line). There was a 10-fold leftward shift of ED_{100}s ($p < 0.0001$) for the average of the EM analogs versus morphine (ED_{100} 0.13 vs 1.4 μg/rat for morphine) (**Fig. 4E**). The AUC for analog 4 was significantly greater than morphine ($p < 0.05$, **Fig.4F**).

Because of the relevance of i.v. drug administration for postoperative pain, we tested EM analogs 1 and 4 for alleviation of postoperative allodynia with the von Frey test for effectiveness after this route of administration. Relief from allodynia after EM analogs 1 (onset 35 min, duration 140) and 4 (onset 35, duration 140) was equal to that of morphine (onset 15, duration 140) as indicated by the solid lines in **Fig. 4G**; dashed lines indicate a significantly greater effect of the EM analogs versus morphine at later time

points. Similar to neuropathic pain experiments, we observed no difference in the DR curves on a mg/kg basis (**Fig. 4H**) but ,when calculated on a molar basis, a significant 1.06x leftward shift (p=0.0028) of the ED_{50} for the EM analogs relative to morphine was observed (ED_{50} 2.8 vs 4.7 μmol/kg for morphine, **Fig. 4I**. AUC was equal for morphine and EM analogs 1 and 4 (**Fig. 4J**).

These data indicate the effectiveness of EM analogs 1 and 4 after central and systemic administration for postoperative pain. As with neuropathic pain experiments, the compounds are more potent centrally, but the equiantinociceptive i.v. doses suggest that morphine equivalent dosing in humans could be an appropriate starting point for these compounds.

Endomorphin analogs dose-dependently block visceral pain.

The number of writhes decreased with increasing doses of morphine (0.18-1 mg/kg) and EM analog 4 (0.018-0.18 mg/kg, **Fig. 5A)**. Effective doses for morphine are in line with previous reports [54,75] and analog 4 was 15 times more potent than morphine. This was the first of our findings where systemic administration of EM analog 4 was significantly more potent than morphine on a mass (mg/kg) basis (ED_{50} 0.02 mg/kg vs 0.30 mg/kg for morphine) (**Fig. 5B**).

Relative potency and efficacy for EM analogs versus morphine

The ED_{50}s (or ED_{100}s for Hargreaves and Randall-Selitto tests) and efficacy (E_{MAX}) for all tests conducted with morphine and the analogs are summarized in **Table 1**. In addition to differences highlighted above, the table illustrates that intrathecal EM analog 4 was essentially equipotent against neuropathic and inflammatory pain as well as postoperative allodynia and thermal hyperalgesia (p > 0.05). By contrast, i.t. morphine was significantly less potent for neuropathic and inflammatory pain relative to mechanical allodynia in postoperative pain (p <0.05), indicating a more consistent response to EM analogs across pain states. In all cases, EM analogs were more potent than morphine. For the intravenous studies, potencies for morphine and EM analogs were generally similar on a mg/kg basis but, on a molar basis, EM analog 1 was more potent than morphine in postoperative pain and analog 4 was more potent in both neuropathic and postoperative pain. For inflammatory pain, morphine was more potent on a mg/kg basis, but not significantly different on a molar basis. Efficacy was similar for morphine and EM analogs across tests.

Discussion

The present study shows that, relative to morphine, the current standard treatment, novel EM analogs, particularly ZH853 (EM analog 4), provide a favorable profile of relief from multiple forms of pain. Tests in well-validated models of neuropathic, inflammatory, postoperative, and visceral pain states conducted here provide a broad, although not exhaustive, assessment of the novel compounds. In all cases, the EM analogs were more potent or equipotent to morphine and their effects were longer lasting.

Because opioids are clinically administered both i.t. and i.v., we investigated both routes. The greatest increase in potency relative to morphine was observed in the models of neuropathic and inflammatory pain, followed by visceral and postoperative pain. Morphine DR curves shifted, depending on the pain model, to a greater degree than ZH853 DR curves did. Thus, the changes in relative potency were due more to the change in morphine potency than that of the EM analogs. Of particular interest is that intrathecal ZH853 was essentially equipotent against neuropathic, inflammatory, and postoperative allodynia pain and significantly more potent than morphine on all three tests. By contrast, morphine was significantly less potent for neuropathic and inflammatory pain relative to postoperative pain. Possible explanations for these differences include 1) greater sensitivity of the morphine response to pain intensity [59] while responses to EM analog 4 are more independent of pain state and test method, and 2) that morphine, but not ZH853, effects on glial activation [91] result in greater variation in morphine responses. The EM analogs were significantly more potent relative to morphine after i.t. administration, indicating greater pharmacodynamic effectiveness. When administered i.v., the EM analogs were about equipotent to morphine but lasted much longer in all pain paradigms. Although the EM analogs penetrate the blood-brain barrier [91], the i.v. results indicate that the penetration is less than that of morphine. The net result of lower penetration but greater central potency is that after i.v. administration, the morphine dose equivalency is near 1. If this translates to humans, it could provide a convenient index for morphine substitution.

Neuropathic pain is among the most difficult types of pain to treat and it is commonly believed that opioids are ineffective against it. Early animal studies produced conflicting results dependent on a variety of factors, including the model used and route of administration, but a more recent consensus is that opioids are generally effective [26,94]. A clinical study reported opioids to be ineffective in patients with neuropathic pain [2].

However, in several studies, opioids have shown efficacy in different types of neuropathic pain [24,25,64,66,69,70] including analgesia at least as great as that observed with “first line” treatments such as tricyclic antidepressants and gabapentin [66]. Opioids are not recommended for first line treatment of neuropathic pain, not for lack of effectiveness, but due to risk of adverse effects [24,25]. Indeed, a standard measure of effectiveness, number needed to treat (NNT) for a 50% improvement, is generally lower for opioids than for first line drugs [28], indicating greater effectiveness. However, recent changes in recommendations for opioids from first line to 2nd/3rd line drugs for chronic pain, including neuropathic [17,28] and inflammatory pain [50], are due largely to the potential risk of abuse and coincide with the rise in opioid overdose deaths. These trends highlight the value of targeting the mu receptor for effective pain relief if the side-effects can be reduced. In this study, both morphine and the EM analogs, after i.t. and i.v. injection, reversed mechanical hyperalgesia and allodynia in rats with chronic neuropathic pain. However, the EM analogs, which show substantially reduced side effects [91], provided significantly longer-lasting analgesia than morphine, indicating potential for both safer and more effective treatment.

Opioids are effective in the treatment of **inflammatory pain** conditions like osteoarthritis (OA) and rheumatoid arthritis (RA) if they are administered under close supervision of a physician [63]. Current guidelines suggest opioids as 2nd or 3rd line treatment or for cases refractory to other treatments. In addition to the risk of dependence and addiction, opioids have the added risk of increasing falls and injury [9,50]. Nevertheless, opioids are used for 58% of patients prior to total knee or hip replacement [50]. The EM analogs may be of particular help for this clinical population because of their lack of both abuse liability and motor impairment compared to morphine [91] and superior analgesia in inflammatory states as shown in the current study.

Postoperatively, morphine has been the gold standard of pain relief for centuries. However, opioid use can cause complications including respiratory depression, excessive sedation, nausea and vomiting and many authorities have recommended using a minimal dose of opioids [14,23,62]. While NSAIDs can be used in conjunction with opioids, especially preoperatively [14], they do not usually provide adequate postoperative relief and are not necessarily safe for cardiac patients. Additionally, in post-surgical contexts such as traumatic brain injury, sedation or disorientation and respiratory depression-induced changes in intracranial pressure

caused by opioids can confound assessment of severity and affect outcomes after head injury. The current study is limited to examining analgesia and the EM analogs lasted much longer than morphine in postoperative pain. Previously we have shown that the compounds do not cause respiratory depression and, in RotoRod tests, do not cause sedation to the point of motor impairment [91], a critical difference for recovering postoperative patients.

While opioids can be employed to treat organic **visceral pain** conditions, their use in this application is limited by untoward side effects and abuse potential [19,32,61]. The current study found that ZH853 produced more potent analgesia in the acetic acid-induced writhing test than morphine by both i.t. and i.v. routes. Analgesia by ZH853 was more potent than analgesia previously observed with peripheral administration in the tail flick assay [91]. This increased potency parallels previous literature indicating that inhibition of acetic acid-induced writhing requires lower doses of morphine [57,67,77] and other EM analogs [4,74] than analgesic tests of cutaneous sensitivity. Mu agonists have been shown to produce analgesia through peripheral mechanisms. Morphine acts peripherally to inhibit acetic acid-induced writhing [67]. Evidence supports the notion that EM1 analogs may similarly act on peripheral mu receptors to inhibit writhing [4,74]. Further study is needed to determine if ZH853 is acting through peripheral and/or central mechanisms to inhibit acetic acid-induced writhing.

In all models tested, the EM analogs produced longer pain relief than morphine. Although many factors may contribute to this difference, two candidate explanations are 1) the remarkable stability of the EM analogs against blood enzymes [91] and 2) the proinflammatory signaling produced by morphine, but not by the EM analogs [91]. Numerous studies indicate that morphine causes glial activation and proinflammatory signaling [5,18,78,83], especially in disease and pain states [8,34,65,78]. In recent studies, acute morphine analgesia was “unmasked” by inhibition of glial activation and proinflammatory cytokines [42], and chronic morphine induced a paradoxical, inflammasome-dependent prolongation of neuropathic pain [34]. By contrast, we recently showed that chronic EM analogs do not produce proinflammatory signaling in conditions where morphine does [91]. This indicates a key mechanism that differentiates our compounds from traditional opioids and renders them unlikely to exacerbate adverse inflammatory processes.

The focus of this paper is the demonstration that, after acute injection, EM analogs are effective at alleviating established pain in a variety of diverse models. Studies beyond the scope of this paper will be required to establish their full clinical potential. These include tests of sustained effectiveness and continued lack of reward effects during longer term administration in pain states, tests in females, operant pain tests [39,87] and effects on recovery [44,71]. Nevertheless, the present results in classical, widely used preclinical pain tests indicate considerable promise for use of the compounds in a variety of pain states.

Neuropathic, postoperative, inflammatory, and visceral pain represent a large percentage of chronic pain sufferers in the US and abroad and, with approximately 1 in 5 adults suffering worldwide, chronic pain is increasingly being viewed as a major public health problem [33]. In addition, studies of human clinical trials for a variety of pain states show that, regardless of analgesic, most patients experience less than 50% pain relief and only a subset of patients obtains full analgesic relief [12,21,29,52,79]. These findings indicate the dire need for improved analgesics. Concurrently however, rates of opioid addiction and overdose deaths have never been higher [11,86], leading to extensive guidelines and debate about limiting and monitoring opioid use [10,22,51]. To address these conflicting trends, there is desperate need for a non-addictive painkiller that has equal or greater effectiveness relative to morphine. We have shown previously that ZH853 has a reduced side effect profile, including a lack of abuse liability in tests that have 95% predictive validity in humans [59,91], and the current study shows that ZH853 will likely work as a reliable, broad-spectrum painkiller for multiple forms of pain.

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*Figure 1. Neuropathic Pain: EM analogs effectively reversed mechanical hyperalgesia and mechanical allodynia in the spared nerve injury model and are equipotent or more potent than morphine. Following i.t. administration in the paw pressure test, EM analogs showed greater duration of antinociception, with EM analogs 1 and 4 lasting significantly longer than morphine (A). The EM analogs were, on average, 78 times more potent than morphine (B). Area under the curve (AUC) was significantly greater for EM analogs 1 & 4 (C). EM analog 4 was selected for i.v. administration with von Frey testing and reversed mechanical allodynia significantly longer than morphine (D). EM analog 4 was equipotent on a mg/kg basis (E), but more potent on a µmol basis (F), and showed a significantly greater AUC (G). Time points at which the drugs produced significant differences (p< 0.05 or greater) from vehicle are shown as solid bars at the top of the graph. Times at which EM analogs produced significant differences from morphine are shown as dashed lines. *, **** = p < 0.05, 0.0001. Number of animals per group are shown in C and G. Error bars indicate SEM.*

Figure 2. Inflammatory Pain, IT drug administration: EM analogs provided greater duration and more potent relief than morphine for thermal hyperalgesia caused by inflammation. All drugs caused dose-dependent reversal of thermal hyperalgesia, as shown in time course data (A). The dose-response curves at 3hr for morphine but not EM analogs 1,2 & 4, showed a decline in efficacy, suggesting more stable, longer lasting pain relief with the EM analogs (B). EM analog 4 showed significantly greater AUCs for 0.1µg and 0.18 µg versus the morphine 10 µg group (C).The average potency of EM analogs for reversing hyperalgesia (100% recovery) was 52 times that of morphine (D). This ratio increased over time as the morphine, but not EM analog, effect declined (B). Animal numbers are shown in C. Error bars indicate SEM.

Figure 3. Inflammatory Pain, IV drug administration: EM analogs provided greater duration and equipotent relief relative to morphine for thermal hyperalgesia caused by inflammation. EM analog 4 showed longer duration of action with latencies significantly greater than morphine at 3 and 4h (A) and, in contrast to declining dose-response curves over time for morphine, EM analog 4 had nearly identical dose-response curves over the entire time of testing (B). Comparison of DR curves for morphine and EM analog 4 at 1 hour revealed no differences (C). Relative to morphine, EM analog 4 was equipotent on a mg/kg basis as well as on a µMol basis. The AUCs were similar for equal doses (D). Number of animals per group are shown in D. Error bars indicate SEM.

*Figure 4 Postoperative Pain: EM analogs provided greater duration of relief of postoperative allodynia and thermal hyperalgesia and are equipotent or more potent than morphine. After IT administration, EM analogs 1 and 4 reversed mechanical allodynia (A) and thermal hyperalgesia (D) significantly longer than morphine and were 20 (B) and 10 (E), times more potent respectively. AUCs for mechanical allodynia reversal were significantly greater for each EM analog compared to morphine (C, F). After IV administration, EM analogs 1 and 4 produced significantly longer antiallodynia than morphine (G) and were equipotent on a mg/kg basis (H), but significantly more potent on a µmol basis (I). Although the EM analogs showed significantly longer reversal of allodynia (G), the AUCs were not significantly different among morphine and the EM analogs (J). Time points at which the drugs produced significant differences (*p< *0.05 or greater) from vehicle are shown as solid bars at the top of the graph. Times at which EM analogs produced significant differences from morphine are shown as dashed lines. Animal numbers are shown in C, F, and J. Error bars indicate SEM.*

Figure 5 Visceral Pain: EM analog 4 provides potent relief of acetic acid-induced visceral pain. The number of total writhes decreased with increasing doses of EM analog 4 and morphine (A) and the percent of maximum possible inhibition (MPI) (1- number of writhes after drug/ number of writhes after vehicle x 100) increased with increasing doses (B). The dose-response curve for EM analog 4 is significantly shifted leftward, indicating greater potency (B). Animal numbers are indicated in (A). Error bars indicate SEM.

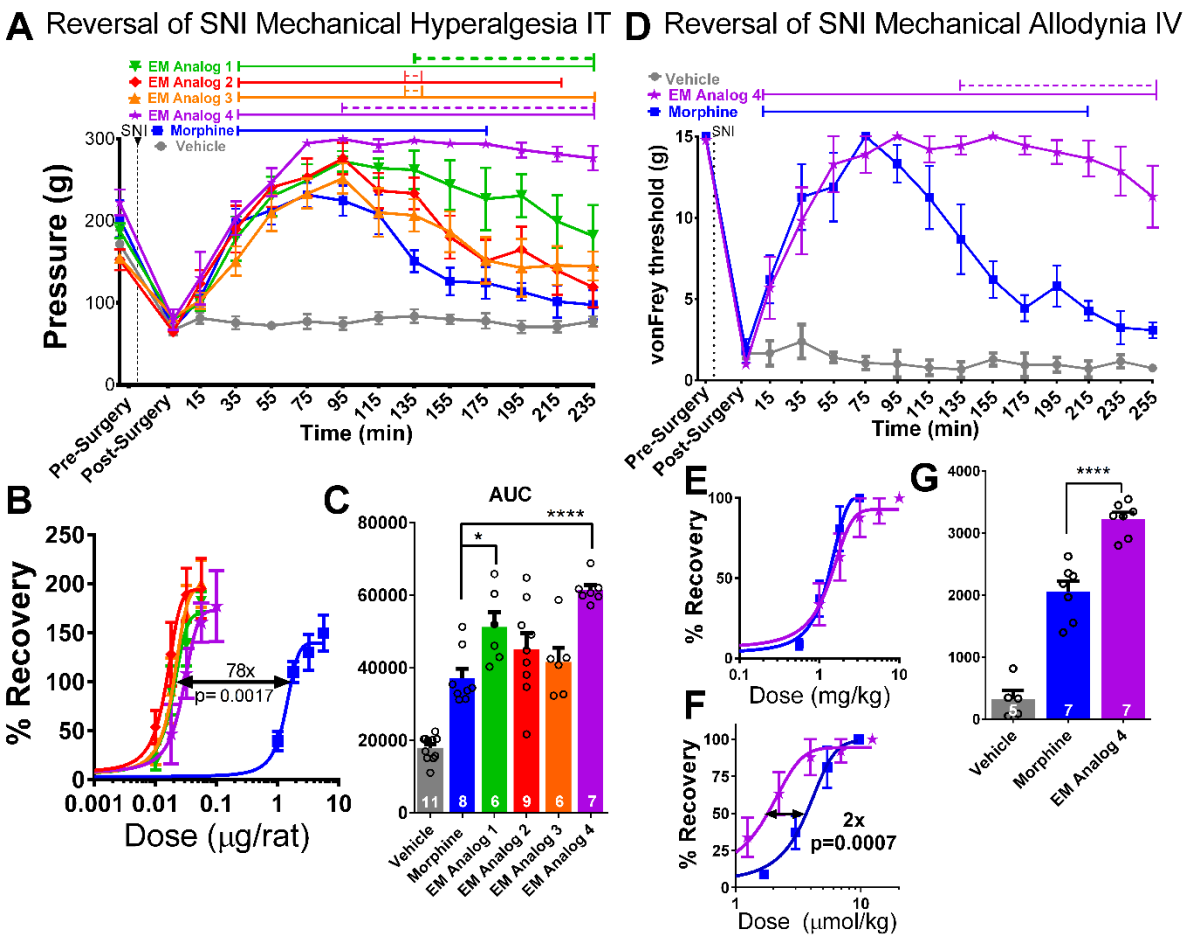
*Table 1. Summary: $ED_{50/100}$, E_{MAX}, and 95% confidence intervals (95% CI) across all pain tests. Route of administration is listed across the top row and is subdivided by pain model and testing method. $ED_{50/100}$ values were calculated by Prism from %MPE or %MPI dose response (DR) curves. For bolus dosing experiments, DR curves from the first hour were used. For **intrathecal administration** (upper panel), all EM analog $ED_{50/100}$ values were significantly left-shifted of morphine and 95% CIs were not overlapping (bolded). Expression as nmol/rat (not shown) results in greater differences in the same direction. When drugs were administered **intravenously** (lower panel), EM analogs and morphine had comparable $ED_{50/100}$ mg/kg values, with the exception of EM analog 4 in inflammatory pain and EM analog 1 in postoperative pain (bolded) which were right-shifted of morphine. When expressed on a molar basis (µmol/kg) however, EM analog 1 showed greater potency in postoperative pain and EM analog 4 was more potent in neuropathic and postoperative pain, and not significantly different for inflammatory pain relative to morphine. E_{MAX} for intrathecal, intravenous, and subcutaneous administration was similar between all EM analogs and morphine, indicating similar efficacy of all drugs on these tests.*

		Intrathecal (µg/rat)			
		Neuropathic (Randal-Selitto)	**Inflammatory (Hargreaves)**	**Postoperative (von Frey)**	**Postoperative (Hargreaves)**
$ED_{50/100}$	EM Analog 1	**0.021**	**0.073**	**0.024**	**0.142**
	EM Analog 2	**0.015**	**0.143**		
	EM Analog 3	**0.020**	**0.064**		
	EM Analog 4	**0.030**	**0.038**	**0.019**	**0.118**
	Morphine	1.669	4.114	0.434	1.351
95% CI	EM Analog 1	**0.017-0.027**	**0.045-0.101**	**0.020-0.028**	**0.079-0.208**
	EM Analog 2	**0.010-0.020**	**0.092-0.207**		
	EM Analog 3	**0.016-0.026**	**0.005-**		
	EM Analog 4	**0.021-0.043**	**0.024-0.056**	**0.015-0.023**	**0.053-0.187**
	Morphine	1.392-2.024	3.018-5.728	0.360-0.500	0.794-1.974
E_{MAX}	EM Analog 1	169.7	234.4	96.1	217.9
	EM Analog 2	194.0	184.1		191.4
	EM Analog 3	196.2	151.6		
	EM Analog 4	172.6	184.6	99.5	203.2
	Morphine	139.4	158.1	98.2	182.7
95% CI_{MAX}	EM Analog 1	135.3-204.2	177.7-291.2	87.29-105.0	161.8-274.0
	EM Analog 2	155.3-232.6	112.1-256.1		127.2-255.6
	EM Analog 3	155.5-237.0	87.68-215.6		
	EM Analog 4	130.9-214.3	139.5-229.7	91.09-107.8	144.8-261.6
	Morphine	119.9-159.0	102.2-214.0	89.82-106.5	140.6-224.8

		Intravenous (mg/kg)			S.C. (mg/kg)
		Neuropathic (von Frey)	Inflammatory (Hargreaves)	Postoperative (von Frey)	Visceral (writhing)
$ED_{50/100}$	EM Analog 1			2.23	
	EM Analog 4	1.46	**5.29**	2.13	**0.02**
	Morphine	1.22	3.01	1.56	0.30
95% CI	EM Analog 1			1.10-2.51	
	EM Analog 4	1.08-1.91	**4.12-7.12**	1.82-2.54	**0.01-0.03**
	Morphine	0.98 - 1.51	2.20-3.96	1.29-1.87	0.15-0.46
E_{MAX}	EM Analog 1			94.70	

	EM Analog 4	94.39	183.60	95.71	91.87
	Morphine	99.40	256.80	90.91	95.02
95% CI_{MAX}	EM Analog 1			84.79-104.6	
	EM Analog 4	82.02-106.8	115.8-251.4	81.51-109.9	80.14-103.6
	Morphine	78.97-119.8	195.0-318.5	80.04-101.8	68.80-121.2
		Intravenous (μmol/kg)			
$ED_{50/100}$	EM Analog 1			**2.97**	
	EM Analog 4	**1.80**	6.52	**2.64**	
	Morphine	3.66	9.02	4.66	
95% CI	EM Analog 1			**2.66-3.34**	
	EM Analog 4	**1.34-2.36**	5.06-8.77	**2.24-3.13**	
	Morphine	2.95-4.53	6.55-11.86	3.87-5.60	

Figure 1: 2 columns



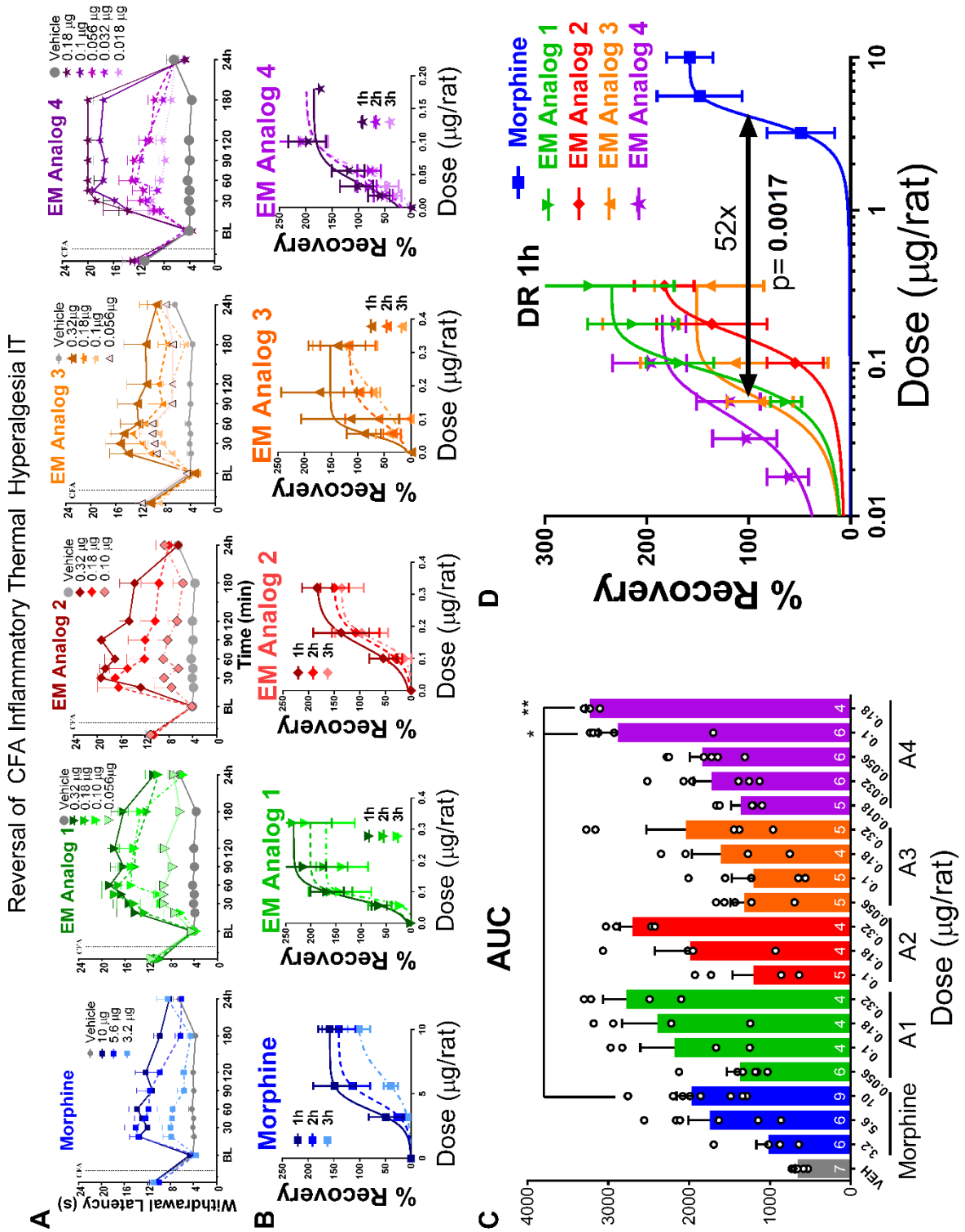
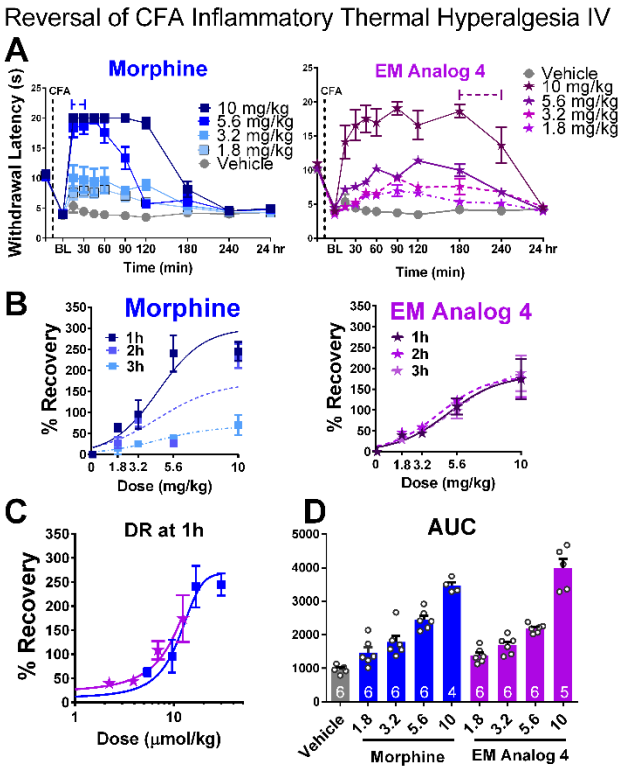


Figure 2: full page

Figure 3: 1.5 columns



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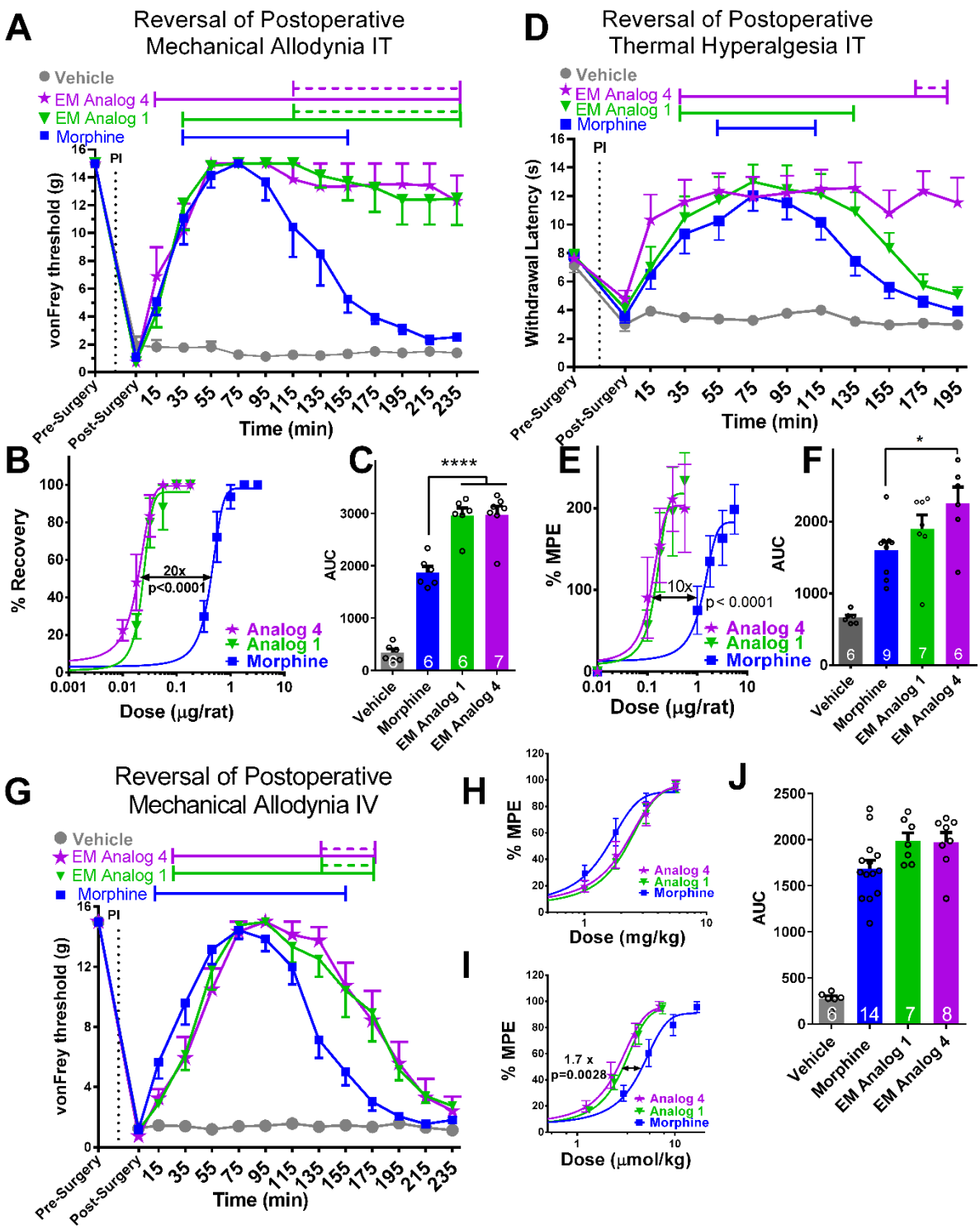

Figure 5: 1 column