Behavioral pharmacology of the mixed-action delta-selective opioid receptor agonist BBI-11008: Studies on acute, inflammatory and neuropathic pain, respiration, GI transit, and drug self-administration

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Conflict of Interest: Edward J. Bilsky is co-owner of BBI. Robin Polt is co-owner of BBI

Abstract

Rationale and Objectives: The studies reported here represent assessment of the behavioral pharmacology of a novel, mixed-action delta-selective (35:1) opioid receptor agonist, BBI-11008. This glycopeptide drug candidate was tested in assays assessing antinociception (acute, inflammatory, and neuropathic pain-like conditions), and side-effect endpoints (respiratory depression, gastrointestinal (GI) transit, and drug self-administration). *Results:* BBI-11008 (3.2 – 100; 10 – 32 mg/kg, i.v.) produced comparable antinociceptive and anti-allodynic efficacy to morphine (1 – 10; 1 – 3.2 mg/kg, i.v.) in assays of acute thermal nociception and complete Freund's adjuvant (CFA)-induced inflammatory pain, and BBI-11008 (1 – 18 mg/kg) had similar efficacy to gabapentin (10 – 56 mg/kg) in a single nerve ligation (SNL) model of neuropathic pain. In the respiration assay, with increasing $\%CO_2$ exposure, BBI-11008 produced an initial increase (32 mg/kg) and then decrease (56 mg/kg) in MV whereas morphine (3.2 – 32 mg/kg) produced dose-dependent decreases only in MV. In the GI transit assay, both compounds produced dose-dependent GI inhibition of transit but the effect was less severe with BBI-11008. In the drug self-administration procedure, BBI-11008 did not maintain self-administration at any dose tested. *Conclusions:* These results suggest that the glycopeptide drug candidate possesses broad-spectrum antinociceptive and anti-allodynic activity with comparable efficacy to the standard prescription opioid morphine. Relative to morphine or fentanyl, the side effect profile for BBI-11008 in the respiration, GI transit, and drug self-administration assays suggests that BBI-11008 may have less

pronounced deleterious side effects. Continued assessment of this compound is warranted.

1. Introduction

Chronic pain remains a major public health concern and represents a spectrum of clinical conditions that are challenging to diagnose and treat (IOM 2011). Standard opioid, adrenergic, steroid, or NSAID drugs used to treat this category of pain are typically fraught with undesirable side effects, and variable efficacy (Antman 2017; Godfrey 1996; Kaye et al. 2017; Krashin et al. 2015). Strategies for drug discovery in the pain field include the elucidation of novel non-opioid targets as well as development of effective but safer mixed-action opioid analgesics or molecules that target non-mu subtypes of the opioid receptor (Fischer 2011; Yekkirala et al. 2017). One particular method of improved opioid drug design has been the synthesis of bivalent compounds that bind to and activate and/or block both mu and delta opioid receptors. This particular mixed structure activity has resulted in lead candidates with maintained or enhanced analgesic efficacy and attenuated side effects relative to typical prescription mu opioid analgesics that contain a relatively narrow therapeutic index (Anand et al. 2015; Bilsky et al. 2000; Elmagbari et al. 2004; Mosberg et al. 2014; Schiller et al. 1995; Stevenson et al. 2015).

Our laboratories and others have synthesized and tested novel mixed-action delta/mu opioid agonists, in an effort to develop effective but safer opioid analgesics (Li et al. 2012; Schiller et al. 1995; Yamamoto et al. 2007). As an example of this, we have reported that the mixed-action delta/mu opioid MMP-2200 has antinociceptive efficacy. This compound was designed to activate mu and delta receptors with equal potency and efficacy. Behavioral readouts indicated measurable but attenuated side effects, including abuse liability and tolerance/dependence, relative to selective mu and/or standard

prescription opioids, in rodents (Lowery et al. 2011; Stevenson et al. 2015). In a continued effort to design and test effective yet safe mixed action ligands, a novel series of compounds was recently developed with greater activity at delta versus mu receptors. Here we report on the lead candidate BBI-11008 for analgesic efficacy in assays of acute, inflammatory and neuropathic pain, as well as receptor mediation, respiratory depression, GI transit, and drug self-administration. BBI-11008 is a bivalent compound based on the dermorphins and deltorphins which are naturally-occurring opioid agonists that are expressed in the skin of frogs and other amphibians. Surprisingly, they are expressed with non-proteogenic D-amino acids. Glycosylation of these compounds results in enhanced penetration of the blood-brain barrier (Erspamer et al. 1989; Heck et al. 1996; Kreil et al. 1989; Melchiorri et al. 1996).

To determine the capacity for broad spectrum analgesic efficacy, BBI-11008 was tested in assays of acute thermal, complete Freund's adjuvant (CFA)-induced inflammatory (Taurog et al. 1988), and SNL-induced neuropathic-like pain conditions (Colburn et al. 1999). Delta vs. mu receptor mediation, and central vs. peripheral activity for BBI-11008 were determined in the acute thermal nociception assay. In order to characterize typical opioid side effects, the delta-selective glycopeptide (~35:1, Figure XX) BBI-11008 was also assessed for efficacy to depress respiration, slow gastrointestinal (GI) motility, and maintain drug self-administration. The analgesic, respiratory, and GI effects of BBI-11008 were compared to the standard prescription opioid agonist, morphine, and for the SNL procedure, BBI-11008 was compared to the voltage-gated calcium channel α_2 subunit modulator, gabapentin (Taylor 2009); in the

self-administration procedure BBI-11008 was compared to the selective mu opioid agonist fentanyl.

Materials and Methods

Subjects. Male CD-1 mice (25-40g, Charles River Laboratories) were used for all acute thermal antinociception, CFA inflammatory tactile allodynia, and GI transit experiments, and male Sprague Dawley rats (250-350g, Charles River Laboratories) were used for all SNL neuropathic model tactile allodynia, respiration, and drug self-administration experiments. Mice were housed in groups of n=4, and rats were housed in groups of n=2-3, in the University of New England Animal Care Facility. All animals received food and water available *ad libitum* and were maintained in a temperature and humidity controlled colony on a 12-h light/dark cycle (lights on at 0700 and off at 1900). Animals were acclimated in the animal facility for at least 5 days prior to use. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and procedures were approved by the University of New England Institutional Animal Care and Use Committee (IACUC). The health of the animals was assessed daily by laboratory technicians and animal care staff.

^{3}H-Binding Studies. Receptor binding studies were done by radioligand displacement studies following published procedures.(Lowery, et al 2011). Chinese hamster ovary (CHO) cells stably transfected with the human δ-opioid receptor (hDOR-CHO), μ-opioid receptor (hMOR-CHO), or κ-opioid receptor (hKOR-CHO) were obtained from Drs. Larry Toll (SRI International, Palo Alto, CA USA) and George Uhl (NIDA Intramural Program, Bethesda, MD USA). The cells were grown in 100 mm dishes in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (10,000 units/mL) at 37 ºC in a 5% CO_2 atmosphere.

CHO cells, expressing either the human MOR, DOR, KOR were incubated with 12 different concentrations of each drug to be assayed, and the radiolabeled ligand in 50 mM Tris-HCl, pH 7.5, at a final volume of 1 ml. Nonspecific binding was measured by the inclusion of 10 μM naloxone. Data were taken as the mean K_i values ± S.E.M. from three separate experiments performed in triplicate. Incubation times of 60 min were used for the MOR-selective peptide [^{3}H]DAMGO and the KOR-selective ligand [3H]U69,593. A 3 hour incubation was used with the DOR-selective antagonist [^{3}H]naltrindole. The IC_{50} values for the glycopeptides were determined using final concentrations of [^{3}H]DAMGO, [^{3}H]naltrindole and [^{3}H]U69,593 of 0.25 nM, 0.2 nM and 1 nM, respectively. Nonspecific binding was measured by the use of 10 μM naloxone to block all opiate binding. The K_i values of unlabelled compounds were calculated from the equation $K_i = IC_{50}/(1+S)$, where S = (concentration of radioligand) / (K_D of radioligand) (Cheng and Prusoff, 1973).

Assay of Thermal Nociception. Antinociception was assessed using a 55˚C warm-water tail-flick test. The latency to the first sign of a rapid tail flick was defined as the behavioral endpoint (Janssen et al. 1963). Each mouse was first tested for baseline latency by immersing its tail in the warm water and recording latency to tail flick. Mice not responding within 5 sec were excluded from further testing. Mice were administered either saline, or a single dose of BBI-11008 (3.2-100mg/kg i.v.) or morphine (1-10mg/kg i.v.) and tested for antinociception at time points, 10, 20, 30, 45, 60, 90, 120, 150 and 180 min post drug administration. Antinociception was calculated using the following formula:

% Antinociception = 100 x (test latency-control latency)/(10-control latency)

To avoid tissue damage, if a subject did not withdraw its tail within 10 sec, the tail was removed from the water by the experimenter, and a latency of 10 sec was recorded for that measurement.

Antagonist Studies. To determine the opioid receptors involved in BBI 11008-mediated thermal antinociception, mice were pretreated with the following antagonists: the nonselective opioid antagonist naloxone (1 mg/kg, s.c.), the peripherally selective opioid antagonist naloxone-methiodide (3.2 mg/kg, s.c.), the mu-selective antagonist β-FNA (19 nmol, i.c.v.), and the delta-selective antagonist naltrindole (10 mg/kg s.c.). Doses and pre-treatment times were based on previous experiments from our laboratories (Lowery et al., 2011). For all pretreatment studies, BBI was injected at t=0 and mice were tested in the tail-flick assay at the 20 min mark. To determine central vs. peripheral opioid receptor mediation, antagonist pretreatment dose and time were as follows: naloxone HCL, 1mg/kg, -10 min; naloxone-methiodide, 3mg/kg, -10min. To determine degree of mu opioid receptor mediation, pretreatment drug, dose, time were as follows: β-FNA, 19nmol, i.c.v., -24 hr. The highly selective, high-efficacy mu agonist LYM-100 (1mg/kg, i.v.) was administered alone and following administration of β-FNA (-24hr), as a positive control. To determine degree of delta opioid receptor mediation, pretreatment drug, dose, time were as follows: naltrindole, 10mg/kg, s.c., -10min. The highly selective, high-efficacy delta receptor agonist DPDPE (30nmol, i.c.v.) was administered alone and administered following naltrindole (-10 min), as a positive control.

CFA Model. Inflammation was induced by a single administration of 20µl CFA (EMD Chemicals, 0.1% dry *Mycobacterium butyricum* dissolved in 85% Drakeol 5NF

and 15% Arlacel A), using a 50µl luer tip glass syringe (Hamilton) with a 30 gauge ½ inch beveled needle, into the subcutaneous space of the plantar surface of the left hind paw in the center of the pads following light anesthesia. Mice were anesthetized with a 3% isoflurane at a flow rate of 0.8-1 L/min with oxygen, for approximately 60-90 sec, until a light depth of anesthesia was attained.

Assay of CFA-induced Tactile Allodynia. To assess tactile allodynia, mice were individually placed into Plexiglas chambers (16 chambers at: 4”x3”x3”, Marine Ecological Habitats) with a wire mesh bottom. This structure was suspended on a PVC pipe frame designed and built by author E.J.B. Mice were allowed to acclimate for 30-60 min, or until exploratory and grooming behavior declined to a level compatible with behavioral testing. A series of monofilaments were applied to the mid-plantar left hind paw (ipsilateral side of CFA injection) that ranged in stiffness from 0.04 to 4 g (0.04, 0.07, 0.16, 0.40, 1, 2, 4). Filaments were applied once for 5 sec with inter-stimulus intervals of 1 min. Mice were tested using the up-down method (Chaplan et al. 1994). Briefly, mice were first tested with the 0.40 g monofilament. A positive response was operationally defined as a rapid withdrawal of the left hind paw or licking of the paw. If the 0.40 g monofilament did not elicit a positive response, the next highest filament in the sequence was tested until the mouse showed a positive response. If the 0.40 g monofilament did elicit a response, the next lowest filament was used until the mouse stopped emitting a positive response. All von Frey experiments were conducted 24 hr after CFA administration with a CFA baseline reading taken prior to drug administration. A decrease in von Frey threshold following CFA relative to control latencies indicated an allodynic response. Either BBI-11008, morphine, or saline was administered after the 24

hr post-CFA administration baseline. Immediately after drug administration, animals were returned to their assigned Plexiglas test chambers and a complete time course for each drug dose was characterized at times 15, 30, 45, 60, 90, and 120 min (or until the test latency approached post-CFA baseline latency) post drug administration.

Neuropathic Pain Model. Rats were anesthetized in an induction chamber with 3-5% isoflurane at a flow rate of 0.8-1L/min with oxygen, for approximately 60-90 sec. Once anesthetized, rats were shaved with hair clippers from the dorsal pelvic area to shoulder blades and placed on a nosecone with 1.5-2% isoflurane. A surgical scrub of alcohol and betadine was applied to the shaven area. Using the level of the posterior iliac crest as midpoint, a 4 cm incision was made in the dorsal midline using a #10 scalpel blade. A midsacral incision was then made and slid approx. 1.5-2 cm along the left side of the spinal wall. Muscle and ligaments were then blunt dissected and retracted, exposing a portion of the spine down to the level of the transverse process. The transverse process of the left L6 vertebra was then carefully nipped off using bone rongeurs (Fine Science Tools #16015-17) to expose the L4/L5 spinal nerves. The L5 nerve was slightly elevated and separated from the L4 nerve using a small custom glass hook ("Chung rod"). The L5 nerve was then ligated using a 4-0 silk suture that was maneuvered around the nerve using a slip-knot that secured the suture on the glass hook. The L6 nerve was then hooked from under the medial edge of the sacrum and gently lifted and ligated in the same manner as the previous L5 ligation. Damaged tissue was then debrided and fascia and muscle were sutured with 3-0 Vicryl suture. The skin was then closed using wound clips and topical triple antibiotic was applied to the wound. The rat also received 100µl i.p. injection of Gentamicin at 10 mg/ml and was allowed to recover from anesthesia

before being placed into a new single housing container where it was allowed to recover for 7 days.

SNL-induced tactile allodynia. To assess allodynia, rats were individually placed into Plexiglas chambers (6 chambers at: 10”x4.5”x6”, Marine Ecological Habitats, Biddeford, ME) with a wire mesh bottom. This structure was suspended on a PVC pipe frame designed and built in house (E.J.B). Rats were allowed to acclimate for 30-60 min, or until exploratory and grooming behavior declined to a level compatible with behavioral testing. A series of monofilaments were applied to the mid-plantar left hind paw (ipsilateral side of CFA injection) that ranged in stiffness from 0.40 to 15 g (0.40, 0.60, 1, 2, 4, 6, 8, 15). Filaments were applied once for 5 sec with inter-stimulus intervals of 1 min. Rats were tested using the up-down method (Chaplan et al. 1994). Briefly, rats were first tested with the 2 g monofilament. A positive response was operationally defined as a rapid withdrawal of the left hind paw or licking of the paw. If the 2 g monofilament did not elicit a positive response, the next highest filament in the sequence was tested until the mouse showed a positive response. If the 2 g monofilament did elicit a response, the next lowest filament was used until the rat stopped emitting a positive response. All von Frey experiments were conducted 7 days after SNL surgery with a new baseline reading taken prior to drug administration. Drug or saline was administered after the 7-day post-SNL baseline. Immediately after drug administration, animals were returned to their assigned Plexiglas test chambers and a complete time course for each drug dose was characterized at times 15, 30, 45, 60, 90, 120, 150, and 180 min (or until the test latency approached post-CFA baseline latency) post drug administration.

Respiration Studies

Apparatus and Procedure. Whole body plethysmography equipment consisted of a Buxco Bias Flow Regulator, Buxco Gas Analyzer, and Buxco Max II. Rats were initially placed in the respiration chambers for 30 minutes to allow for habituation. Following the 30-minute habituation, rats were subcutaneously injected with either saline, morphine (3.2 - 32 mg/kg), or BBI-11008 (32 - 56 mg/kg). Total session duration was 115 minutes. Respiratory parameters recorded included respiratory frequency (breaths per minute = f_R), tidal volume (volume inhaled = V_T), and minute volume ($MV = f_R \times V_T$). Minute volume (MV) is the rate of ventilation and represents the amount of gas exhaled by each rat, per minute. CO_2 was raised to 4% at 75 minutes, 6% at 90 minutes, and 8% at 105 minutes, with 5 min CO_2 purge immediately prior to each increase (eg: CO_2 purge at 70 – 75 min; 85 – 90 min; 100 – 105 min).

The data were averaged over 50 breaths and stored on the computer for analysis. The slope of the hypercapnic ventilatory response was determined from the slope of the relationship between V and the four levels of inspired carbon dioxide using least-squares regression analysis.

GI Transit. Mice were fasted approximately 20 hr prior to testing. At time 0, BBI-11008 (10-180 mg/kg) or morphine (1-10 mg/kg) was administered, i.v. At post-drug administration time X min (time of peak effect for BBI = 20 min; morphine = 30 min), an oral charcoal suspension was delivered using an 18 gauge curved gavage needle (Popper & Sons) on a 1cc syringe. The suspension was made the day of use at 10% charcoal (100-400 activated mesh, Sigma) with a 2.5% arabic acid (Sigma) in distilled water and mixed

thoroughly and repeatedly to minimize needle obstruction. A constant volume of 250 ul charcoal suspension was administered per animal. Animals were sacrificed at 30 minutes post charcoal administration by cervical dislocation. The small intestine (duodenum to cecum) was dissected out and carefully uncoiled. The distance covered, in centimeters, by the charcoal was measured and compared to the total length of the small intestine for each animal.

Percent gastrointestinal transit was expressed by the following formula:

%GI transit = [(distance traveled by the charcoal) ÷ (total intestinal length)] * 100

%GI inhibition = [(% GI transit control - % GI transit compound) ÷ (% GI transit control)] * 100

Drugs. BBI-11008 was synthesized in the Polt laboratory. Morphine sulfate was generously provided by Mallinckrodt (St. Louis, MO). All drugs were dissolved in sterile saline (0.9% NaCl).

Drug Self-Administration. All studies were conducted in drug self-administration operant conditioning chambers (Med Associates, model MED-008-CT-B1) placed within sound-attenuating cubicles equipped with a house light and exhaust fan. Each chamber contained two response levers situated on the front wall of the chamber. A shallow steel cup situated between the two levers, and just above the floor, contained a reservoir for consumption of food pellets. A pellet dispenser delivered 45 mg food pellets (see food training). Stimulus lights were situated above each response lever and were programmed to signal the availability of drug or food. A drug infusion pump was mounted outside each individual chamber to deliver intravenous drug via Tygon tubing. A complete

swivel system with tether (Camcaths, Cambridgeshire, GB) was mounted inside each chamber to allow for unconstrained movement of the animal.

Food training. Lever pressing was initially shaped during daily training sessions (30min day 1; 15 min days 2-8). Food training involved reinforcement of successive approximations of lever-press behavior with delivery of a food pellet. Once shaping was complete, 45 mg food pellets (Noyes brand) were available under a Fixed Ratio (FR) 1 schedule of reinforcement. Illumination of the stimulus light above the active lever served as a discriminative stimulus that the response-food delivery contingency was in place. Responding on the other inactive lever was counted but had no programmed consequences (animals were counterbalanced such that the left lever was active for half the rats and the right lever was active for the other half). A maximum of 50 food reinforcers was available during each daily training session. Once responding stabilized under the FR1 schedule (three consecutive days in which response rates varied by no more than 20% and at least 80% of responses emitted on the active lever), the FR requirement was raised to FR5 over the course of 3-6 sessions. After responding stabilized under the FR5 schedule, using the same criteria as above, rats underwent surgery for implantation of an i.v. catheter.

Surgery. Rats underwent surgery using aseptic techniques. In brief, animals were sedated with a 10-minute pretreatment of midazolam (5mg/ml i.p.) and then anesthetized with an isoflurane/oxygen vapor mixture and implanted with a chronic indwelling i.v. catheter into the right external jugular vein. The surgical procedure was based on

methods described elsewhere (Thomsen and Caine 2005). A single dose of the analgesic non-steroidal-anti-inflammatory drug ketoprofen (5 mg/kg s.c.) as well as the antibiotic amikacin (10 mg/kg s.c.) was administered immediately prior to surgery. Following surgery, animals were allowed to recover for seven days before training was resumed.

Drug self-administration training. After 7 days recovery from surgery, the high efficacy mu opioid agonist fentanyl was available as the reinforcer during three-hour sessions five days/week. Sessions began with a noncontingent "priming" infusion of the available drug dose in a 56µl volume. Responding on the initial food-trained lever was reinforced under a FR1/time out 20 sec schedule with an i.v. infusion of fentanyl (0.0032 mg/kg/infusion). Fentanyl was available until baseline criteria were met (three consecutive sessions with a minimum of 15 reinforcers earned, no more than 20% variation in number of reinforcers earned between three sessions, at least 80% responses on the active lever). Once responding stabilized under the FR1 schedule, the response requirement was raised to FR5 over the course of 4-7 sessions. Following stable fentanyl self-administration under the FR5 schedule, saline substitution was initiated for 1-3 sessions until responding decreased to at least 50% of fentanyl-maintained baseline rates. Baseline fentanyl responding was then re-established followed by determination of a full dose-effect function (see below) using a within-subjects design.

Drug testing. All test sessions began with a non-contingent "priming" infusion of the available drug dose. Test sessions were conducted no more than twice each week and were separated by at least 48 hr. On Mondays, Wednesdays and Fridays, the solution

available for self-administration was either saline or the training dose of fentanyl (0.0032 mg/kg/infusion), and substitution doses were tested on Tuesdays and Thursdays. Following determination of the fentanyl dose-effect function (0.00032 - 0.01 mg/kg/infusion), a range of doses of the mixed action delta/mu opioid agonist BBI-11008 (0.032 - 3.2 mg/kg/infusion) was tested. The primary dependent variable for drug self-administration was number of drug infusions. Statistical analysis was accomplished with one-factor ANOVA. A significant ANOVA was followed by the Duncan post hoc test. Significance was set a priori at $p \leq 0.05$.

Results

Effects of BBI-11008 and morphine on acute thermal nociception. Figure 2 shows the effects of BBI-11008 (3.2 – 100 mg/kg, iv) and morphine (1-10 mg/kg, iv) in the thermal tail flick assay in mice. Both BBI and morphine produced dose- and time-dependent thermal antinociception. Within the dose ranges tested, BBI peak antinociception occurred for ~30 min, whereas morphine peak antinociception occurred for ~ 60 min, with overall duration of effect lasting ~90 min and ~120 min, respectively. Duration of effect for peak doses was quantified by Area under the Curve and BBI 100 mg/kg (AUC: 5936) was ~54% of morphine 10 mg/kg (AUC: 10855). It should be noted that the top dose of morphine used was supra-maximal under the conditions examined.

Antagonism studies. Figure 3 shows antagonism studies in the tail flick assay in mice. The left-most panel shows central vs. peripheral receptor mediation of BBI-11008. The opioid antagonist naloxone (1mg/kg) blocked BBI-induced thermal nociception ($p \leq 0.001$). In contrast, the peripherally restricted naloxone methiodide (3mg/kg) was ineffective in blocking BBI-11008 effects. The middle panel shows mu receptor mediation. The selective mu opioid antagonist β-FNA (0.1 mg/kg) only partially blocked the effects of BBI-11008 in the tail flick assay ($p \leq 0.001$), but as expected, fully blocked the effects of the positive control and selective mu agonist LYM (1 mg/kg; $p \leq 0.001$). The right-most panel shows delta receptor mediation. The highly selective delta antagonist NTI (1 mg/kg) fully blocked the effects of BBI-11008 as well as the positive control and selective delta agonist, DPDPE, in the tail flick assay ($p \leq 0.001$).

Effects of BBI-11008 and morphine on CFA-induced tactile allodynia. Figure 4 shows the effects of BBI and morphine on CFA-induced tactile allodynia in mice. Both BBI-11008 (10-32 mg/kg, iv) and morphine (1-3.2 mg/kg, iv) produced dose- and time-dependent reversal of tactile allodynia in the von Frey assay. Both compounds produced peak effects at 15-30 min, with duration of action slightly longer for BBI (compare 32 mg/kg BBI with 3.2 mg/kg morphine at 45 min). Duration of effect for peak doses was quantified by Area under the Curve and BBI 32 mg/kg (AUC: 18.18) was approximately 1.4-fold greater than morphine 3.2 mg/kg (AUC: 12.85).

Effects of BBI-11008 and morphine on SNL-induced tactile allodynia. Figure 5 shows the effects of BBI-11008 and Gabapentin on SNL-induced tactile allodynia in rats. Both BBI (1-18 mg/kg, iv) and Gabapentin (10-56 mg/kg, iv) produced dose- and time-dependent reversal of tactile allodynia in the von Frey assay with BBI showing an earlier onset of action relative to gabapentin (compare at 30-min mark), but gabapentin showing longer duration of action (compare 90-150 min). Duration of effect for peak doses was quantified by Area under the Curve and BBI 18 mg/kg (AUC: 116.8) was ~90% of gabapentin 56 mg/kg (AUC: 130.2).

Effects of BBI-11008 and morphine on minute volume with increasing CO_2 exposure. Figure 6 shows the effects of BBI-11008 (32, 56 mg/kg, iv) and morphine (3.2-32 mg/kg, iv) on % control minute ventilation (MV) under control conditions (0), and at 4, 6, and 8% CO_2 exposure in rats. For both compounds, minute ventilation was increased with increasing CO_2 exposure. The left panel shows that, with increasing CO_2

exposure, 32 mg/kg BBI produced increases in MV relative to saline controls, and a higher dose of 56 mg/kg produced decreases in MV relative to saline controls (Interaction: $F = 10.97$, $df = 6/60$, $p < 0.0001$; Dose: $F = 74.40$, $df = 2/60$, $p < 0.0001$; $\%CO_2$: $F = 148.10$, $df = 3/60$, $p < 0.0001$). The right panel shows that, with increasing CO_2 exposure, morphine produced dose-dependent decreases in MV relative to saline controls (Interaction: $F = 15.62$, $df = 9/80$, $p < 0.0001$; Dose: $F = 70.02$, $df = 3/80$, $p < 0.0001$; $\%CO_2$: $F = 279.23$, $df = 3/80$, $p < 0.0001$).

Effects of BBI-11008 and morphine on GI transit. Figure 7 shows % GI transit for BBI-11008 and morphine in mice. The left panel shows that BBI-11008 (10-180 mg/kg) produced a dose-dependent inhibition of GI transit with the highest dose of 180 mg/kg producing ~50% inhibition of transit, relative to vehicle ($F = 14.43$, $df = 4$, $p < 0.0001$). The right panel shows that morphine also produced a dose-dependent inhibition of GI transit, but the magnitude of inhibition was more marked with the highest dose tested (10 mg/kg) producing ~ 83% inhibition of GI transit, relative to vehicle ($F = 54.74$, $df = 4$, $p < 0.0001$).

Self-Administration of Fentanyl and BBI-11008. Figure 8 shows drug self-administration data for the reference mu opioid agonist fentanyl (0.00032 – 0.01 mg/kg/infusion) and BBI-11008 (0.032 – 3.2 mg/kg/infusion). Fentanyl produced a characteristic inverted U-shaped dose-effect function. ANOVA revealed that fentanyl (0.001 and 0.0032 mg/kg/infusion) maintained significantly greater responding than saline ($F = 7.386$; $df = 4,42$, $p < 0.001$). In contrast, BBI-11008 did not maintain drug

self-administration at any dose tested and the full range of BBI 11008 doses were not significantly different from saline.

4. Discussion

The present paper represents an assessment of the behavioral pharmacology of the novel, mixed-action delta/mu opioid receptor agonist, BBI-11008 in rodents. The main findings were that BBI showed similar analgesic efficacy to the prescription opioid agonist morphine, but showed a slightly improved side effect profile, manifested as attenuated respiratory depression, less pronounced GI slowing, and absence of drug self-administration. An additional finding was that the acute antinociceptive effects of BBI were mediated by both delta and mu opioid receptors, as a mu receptor antagonist produced partial reversal of BBI-mediated thermal nociception. In vitro studies with BBI-11008 (unpublished) indicated that it was more selective for DOR over MOR (compared to MMP-2200), though the present in vivo data indicate there is still a mu component contributing to the pharmacology. Finally, the mu-mediated effects were determined to be centrally, but not peripherally, mediated.

The broad spectrum analgesic efficacy of BBI-11008 was tested in an assay of acute thermal nociception, and in models of CFA-induced inflammatory pain (Taurog et al. 1988), and SNL-induced neuropathic pain (Colburn et al. 1999). In the thermal nociception tail withdrawal test, BBI displayed antinociceptive efficacy comparable to morphine, but with a shorter duration of peak effect (~30 min) compared to morphine (~1 hr). In the CFA model of tactile sensitivity, BBI had efficacy comparable to morphine with peak effects of both compounds at ~30-45 min. In the SNL model of tactile sensitivity, BBI produced comparable effects to the α_2 sub-unit modulator, Gabapentin, with both compounds showing peak effects at ~90 min and BBI showing a longer duration of action in the SNL neuropathic pain-like model compared to the CFA

inflammatory pain-like model. Overall, peak effects of BBI were ~10-fold less potent than morphine in the acute thermal and CFA assays, and ~5-fold less potent than morphine in the SNL assay. In summary, the nociceptive, and tactile allodynia tests suggest that BBI has comparable efficacy to morphine in thermal acute, inflammatory, pain-like conditions, but is less potent in producing these antinociceptive and anti-allodynic effects.

The behavioral pharmacology data for BBI-11008 reported here, are consistent with the therapeutic/side-effect profiles of mixed-action delta/mu opioid agonists in the literature. For example, our laboratories have previously shown that the delta/mu opioid glycopeptide agonist, MMP-2200 has antinociceptive efficacy similar to morphine in the acute thermal tail withdrawal assay, and that this effect was blocked by selective mu or delta antagonists, suggesting a combined mu/delta mechanism of action. In contrast to morphine, MMP-2200 produced less robust locomotor activity activation, less pronounced naloxone-precipitated withdrawal behaviors, and lower rates of drug self-administration (Lowery et al. 2011; Stevenson et al. 2015). Similarly, Jutkiewicz and colleagues have shown that VRP26, a mixed-action mu agonist/delta antagonist produced equivalent antinociceptive efficacy to fentanyl in the warm water tail withdrawal assay, but in contrast to fentanyl showed significantly less behavioral signs of withdrawal, and no conditioned place preference (Anand et al. 2016).

The receptor mediation of BBI-11008 for thermal antinociception was characterized using the highly selective mu receptor antagonist βFNA, and the selective delta

antagonist, NTI. NTI produced more robust reversal of BBI-induced acute antinociception than βFNA, suggesting that the acute antinociceptive effects of the compound are mediated by both delta and mu receptors, with perhaps greater efficacy derived from delta receptor activation. Further, the degree of antinociception induced by the positive control DPDPE was blocked by NTI to a similar degree, indicating that BBI has affinity and efficacy for delta receptors. Finally, determination of peripheral vs. central mechanism was accomplished by pretreatments with the centrally penetrant mu receptor blocker naloxone, as well as the peripherally restricted naloxone-methiodide. Results indicated that antinociceptive effects are centrally mediated given the effectiveness of naloxone, but not it's methiodide analogue, in reversing BBI-induced antinociception. The central mediation suggests that other routes of administration (other than i.v. reported here) may produce therapeutic effects, and thus further testing of this compound is warranted.

Within the range of doses tested in respiration under normal O_2 levels, BBI-11008 showed comparable respiratory depression to the prescription drug morphine. However, the two compounds showed divergent dose-dependent effects when tested in an environment with increasing $\%CO_2$, with BBI showing increased respiration at one dose, and morphine showing no change or decreased respiration, relative to vehicle controls. The respiration data for BBI add to the complex opioid respiration literature that includes reports of mixed-action delta/mu drugs, dose addition studies using combinations of delta + mu ligands, and separate mu or delta compounds administered alone. Overall, delta agonism as well as antagonism have been reported to attenuate or mediate respiratory

depression and hypercapnia induced by selective mu or delta agonists (Dahan et al. 2001; Lonergan et al. 2003; Morin-Surun et al. 1984; Pazos et al. 1984; Su et al. 1998; Wojciechowski et al. 2011).

In the GI transit assay, both BBI-11008 and morphine produced dose-dependent slowing of GI movement. However, BBI was less potent than morphine, and in the dose ranges tested, BBI produced less pronounced % inhibition (max of ~30% transit) compared to morphine (max of ~5% transit). These data are also consistent with the delta/mu literature in which reports indicate less severe GI paralysis with mixed action compounds relative to selective mu agonists (Wade et al. 2012). However, although central and peripheral mediation of intestinal transit via mu receptor activation is well documented, the role of the delta receptor is less clear (Porreca et al. 1986; reviewed in Meerveld et al. 2004). Specifically, there are examples of delta agonists that produce little or no GI slowing. For example, the selective delta agonist JNJ produced a maximum GI slowing of 67%, compared to morphine which produced a maximum GI slowing of 11% in mice (Codd et al. 2009) and earlier reports showed that delta agonists had no effect on (i.c.v, or s.c. route) or actually enhanced (by i.t. route) GI slowing depending on route of administration (Burks et al. 1988; Galligan et al. 1984). Consistent with delta mediated slowing, though, Wade and colleagues (2012) showed that the mixed mu agonist-delta antagonist "MuDelta" had significantly less GI transit inhibition than selective mu agonists alone. Thus, although the role of delta receptors in GI transit is controversial, the present results with BBI indicate that a mixed-action delta/mu agonist profile does seem to lessen the severity of GI inhibition.

Importantly, respiratory depression and GI slowing/paralysis are frequent and sometimes severe deleterious side effects of clinically prescribed opioids such as fentanyl and morphine, and in some cases, can lengthen hospital stays for patients on high dose maintenance therapy (Fishman et al. 2004). Going forward, it will be important that current efforts in opioid drug design take into consideration the severity of these side effects and focus on structure-activity modifications that may be able to attenuate these (and other) deleterious outcomes. Only if side effect profile can be decreased, will opioid drug discovery continue to be a viable option.

The abuse liability of BBI-11008 was quantified and compared to the mu opioid prescription agonist fentanyl using a drug self-administration procedure. Fentanyl produced the characteristic inverted U-shaped dose-response function seen with selective mu receptor and prescription opioids (Mello and Negus 1996). In contrast BBI did not produce self-administration across a broad range of doses. Although preliminary, these data suggest that it is unlikely that BBI 11008 is rewarding or has abuse liability in humans. However, it is worth noting that a more complete assessment of abuse liability may require monitoring the subjective effects of BBI-11008 in humans after clinically relevant route and formulation are determined and/or further preclinical work using drug discrimination learning procedures (Lynch et al. 2010).

Taken together, BBI-11008 represents a mixed-action delta/mu opioid receptor agonist with comparable antinociceptive efficacy to morphine and gabapentin in assays or models of acute, inflammatory and neuropathic pain. The respiration, GI and self-administration data suggest that BBI-11008 may be safer, relative to prescription opioids such as morphine or fentanyl. Given that respiratory depression, GI slowing, and abuse liability are highly prevalent and serious side effects of prescription mu opioid agonist administration in clinical settings (Fishman et al. 2004), the side effect profile of BBI suggests that this compound may have clinical utility, and further characterization of this drug is warranted.

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Figure captions

Fig. 1. Structure of BBI-11008

Fig. 2. Dose- and time-effect curves for BBI-11008 and morphine in a warm water (50˚) tail withdrawal assay.

Fig. 3. Receptor mediation in a warm water tail withdrawal assay. Left panel shows (left to right) BBI-11008 alone, mu antagonist naloxone + BBI-11008, and peripherally restricted naloxone methiodide + BBI-11008. *** indicates significantly decreased compared to BBI-11008 alone ($p \leq 0.0001$). Middle panel shows (left to right) BBI-11008 alone, selective mu antagonist βFNA + BBI-11008, selective mu agonist LYM 100 alone, and βFNA + LYM 100. *** indicates significantly decreased compared to BBI-11008 alone ($p \leq 0.0001$). ^^^ indicates significantly less than βFNA + BBI-11008 ($p \leq 0.0001$). Right panel shows (left to right) BBI-11008 alone, selective delta antagonist NTI + BBI-11008, selective delta agonist DPDPE alone, and NTI + DPDPE. *** indicates significantly decreased compared to BBI-11008 alone or DPDPE alone ($p \leq 0.0001$).

Fig. 4. Dose- and time-effect curves for BBI-11008 and morphine in a von Frey test of tactile allodynia following CFA administration.

Fig. 5. Dose- and time-effect curves for BBI-11008 and gabapentin in a von Frey test of tactile allodynia following SNL surgery.

Fig. 6. Mean % control minute ventilation under increasing CO_2 exposure for BBI-11008 (left panel) and morphine (right panel). * indicates significantly different compared to saline ($p \leq 0.05$). ** indicates significantly different compared to saline ($p \leq 0.001$). *** indicates significantly different compared to saline ($p \leq 0.0001$).

Fig. 7. %GI transit for BBI-11008 (left panel) and morphine (right panel). ** indicates significantly different compared to saline ($p \leq 0.001$). *** indicates significantly different compared to saline vehicle ($p \leq 0.0001$).

Fig. 8. Dose-effect curves for IV self-administration of BBI-11008 and fentanyl under a FR5-TO-20 sec schedule of reinforcement. ** indicates significantly greater than saline ($p \leq 0.001$). *** indicates significantly greater than saline vehicle ($p \leq 0.0001$).

Fig 1: Acute thermal (Tail flick)

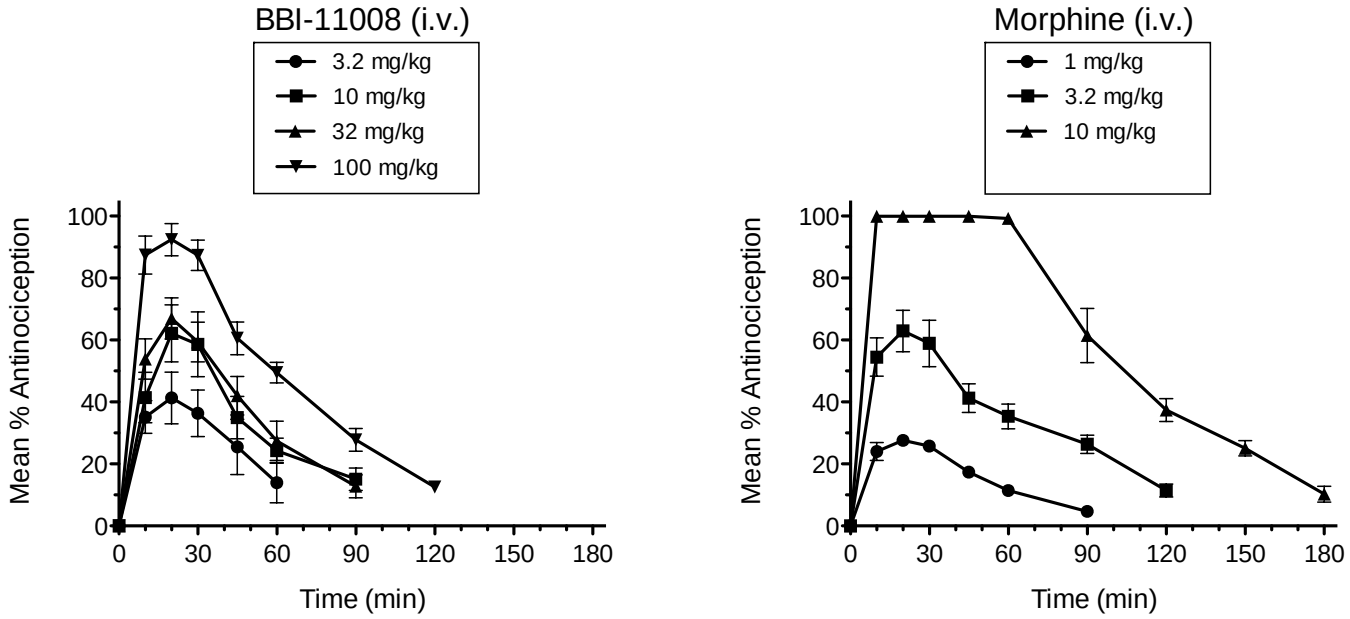


Fig 2: Antagonism studies

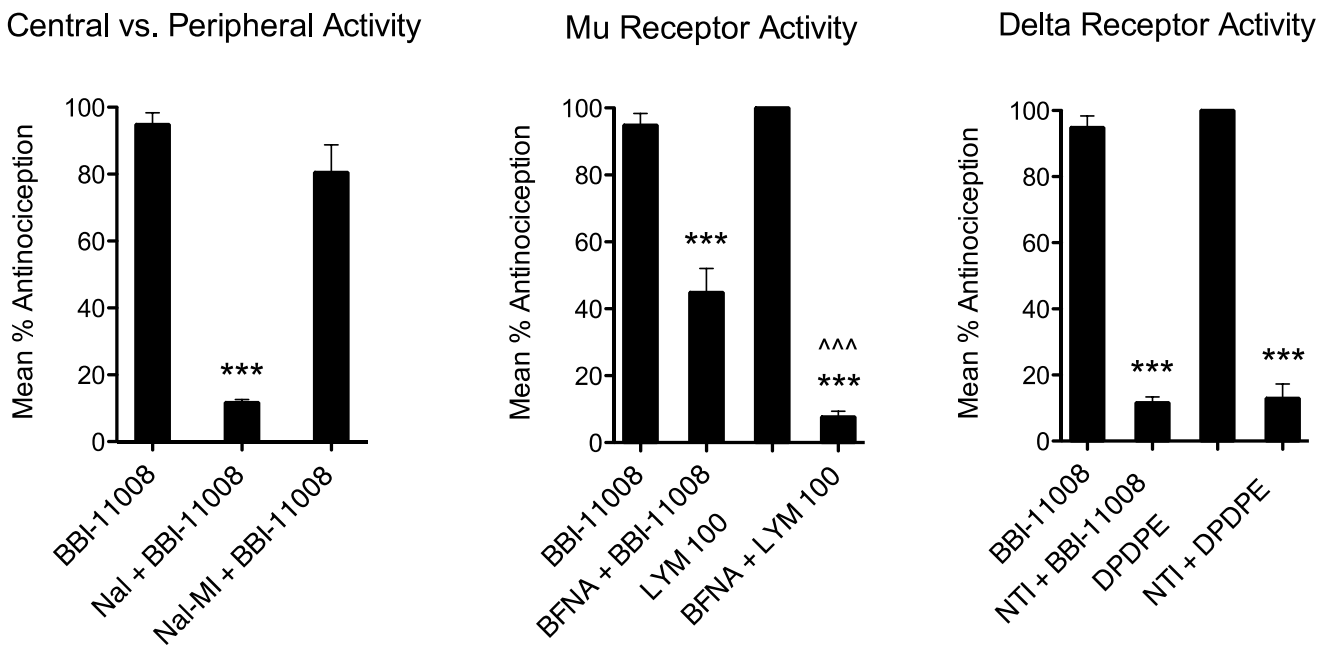


Fig 3: CFA tactile (von Frey)

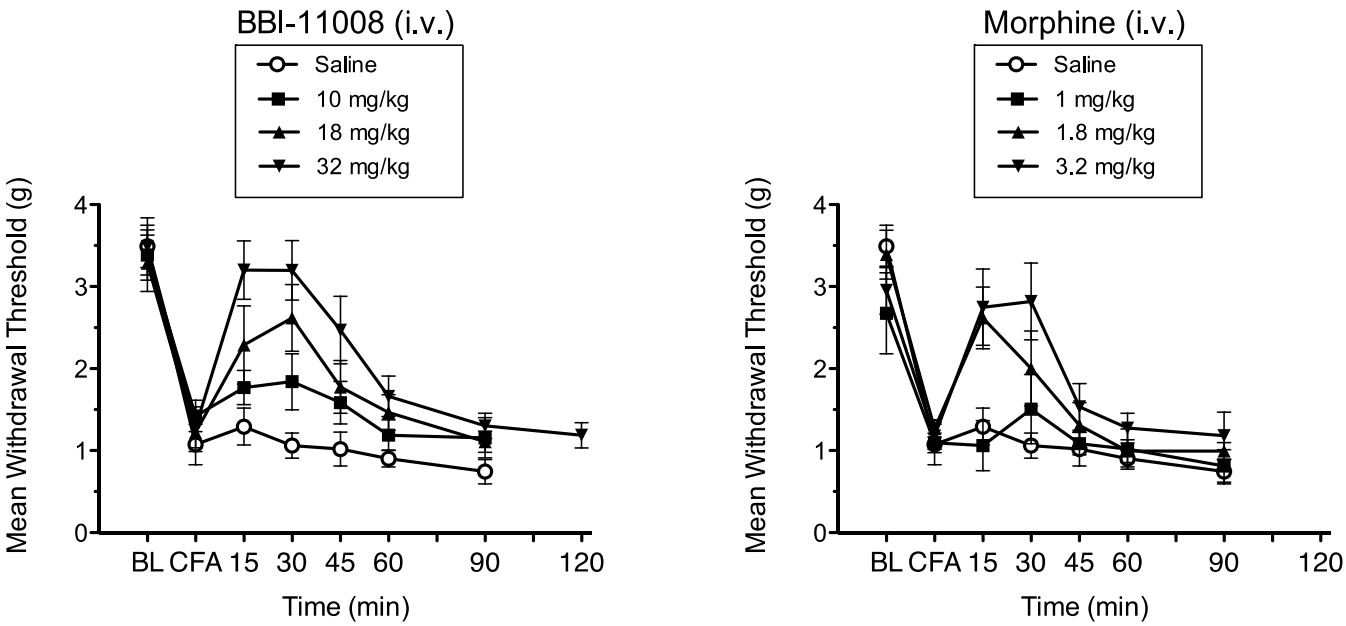


Fig 4: SNL tactile (von Frey)

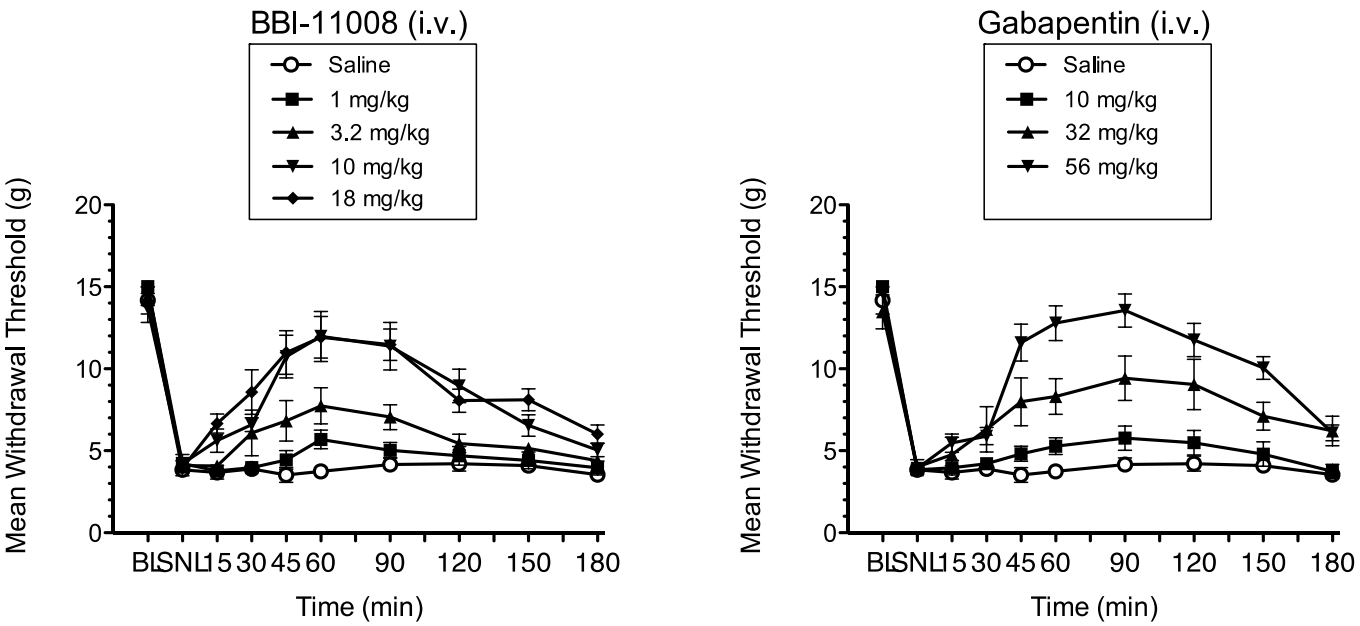


Fig 5: Respiration + CO_2

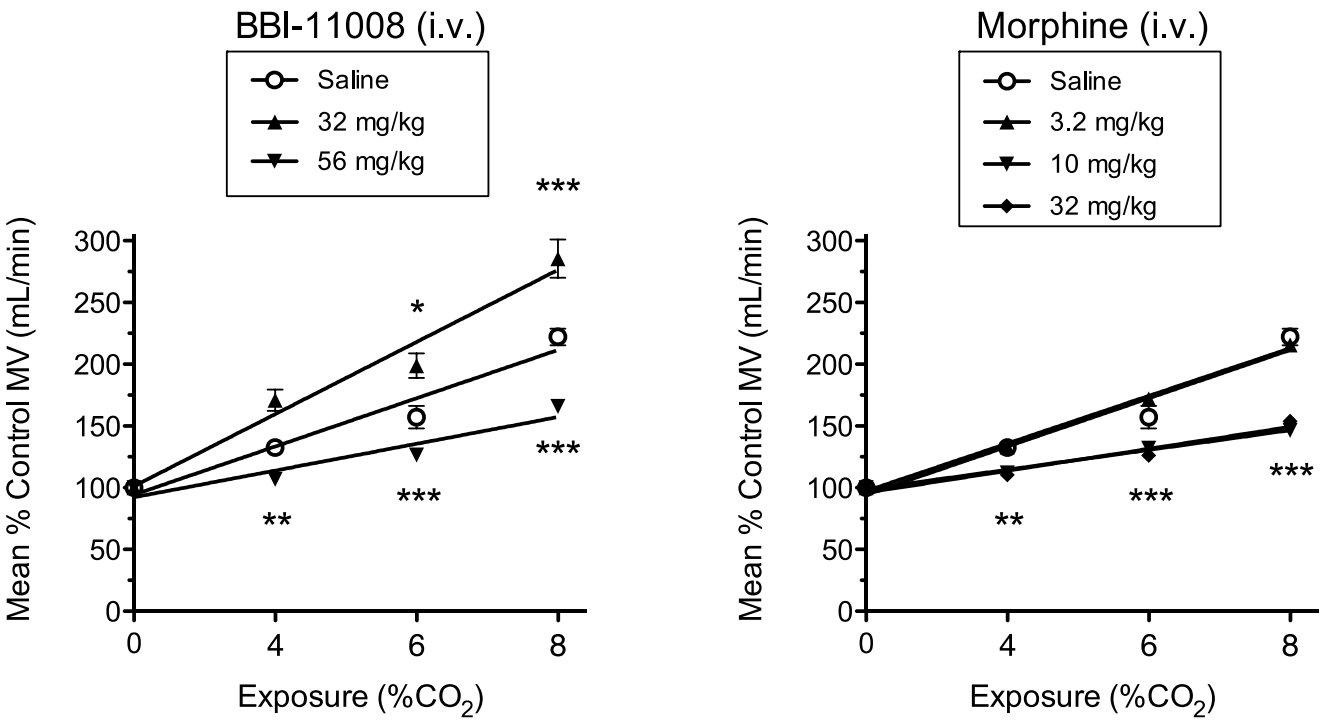
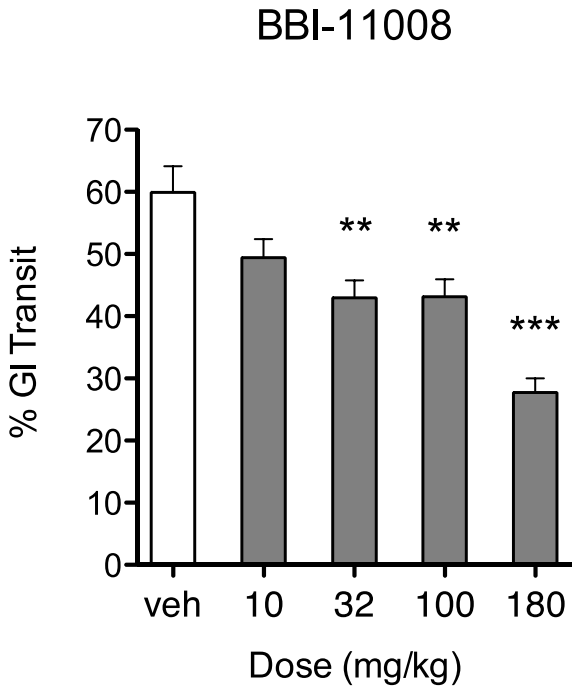


Fig 6: % GI transit



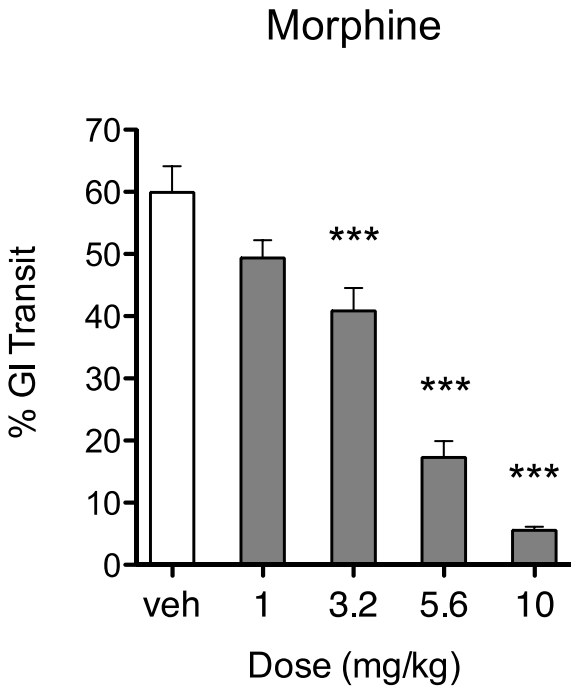


Fig 7: Drug Self-Administration