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In Vivo Effects of μ Opioid Receptor Agonist/δ Opioid Receptor Antagonist Peptidomimetics Following Acute and Repeated Administration

Running Title: μ-Receptor Agonist/δ-Receptor Antagonist Peptidomimetic Effects In Vivo

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ABSTRACT

Background and Purpose

Mu opioid receptor (μ-receptor) agonists are used for pain management, but produce adverse effects including tolerance, dependence, and euphoria. The co-administration of a μ-receptor agonist with a delta opioid receptor (δ-receptor) antagonist has been shown to produce antinociception with reduced development of some side effects. We characterized the effects of three μ-receptor agonist/δ-receptor antagonist peptidomimetics *in vivo* after acute and repeated administration to determine if this profile provides a viable alternative to traditional opioid analgesics.

Experimental Approach

Three μ-receptor agonist/δ-receptor antagonist peptidomimetics, **AAH8, AMB46,** and **AMB47,** and morphine were evaluated for the development of tolerance and dependence after five days of twice daily treatment with escalating doses of drug (10-50 mg kg^{-1}). Antinociceptive effects were measured in the warm water tail withdrawal (WWTW) assay before and after repeated drug treatment; physical dependence was evaluated by naltrexone-precipitated withdrawal jumping. The rewarding effects of **AAH8** were evaluated using a conditioned place preference (CPP) assay with twice daily conditioning sessions performed for five days.

Key Results

Morphine, **AAH8, AMB47,** and **AMB46** all demonstrated acute antinociceptive effects, but repeated administration only produced tolerance in animals treated with morphine and **AMB46.** Injection of naltrexone precipitated fewer jumps in mice treated repeatedly with **AAH8** as compared with morphine**, AMB47**, or **AMB46.** Conditioning with morphine, but not **AAH8**, produced significant CPP.

Conclusions and Implications

AAH8 may be a better alternative than traditional opioid analgesics, producing antinociception with less development of tolerance and dependence and may be less rewarding than morphine.

KEY WORDS: Mixed efficacy, mu opioid receptor, delta opioid receptor, tolerance, dependence, conditioned place preference, peptidomimetic

ABBREVATIONS: **[^{35}S]GTPγS**, [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate; **δ-receptor,** delta opioid receptor; **κ-receptor,** kappa opioid receptor; **μ-receptor,** mu opioid receptor; **BID,** bid in die (twice daily)**; CPP**, conditioned place preference; **DAMGO**, D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; **DPDPE**, D-Pen^{2,5}- enkephalin; **EDTA,** Ethylenediaminetetraacetic acid; **MPE,** maximal percent effect; **NLX,** naloxone; **NTI,** naltridnole; **NTX,** naltrexone; **Ke,** potency of a pure antagonist; **Ki,** inhibition constant for a ligand; **TFA,** trifluroacetic acid; **TST,** tail suspension test; **w/v,** weight to volume; **w/w,** weight to weight **WWTW**, warm water tail withdrawal; **+/+,** wildtype; -/- homozygous knockout.

Introduction

While opioid drugs have significant clinical utility in treating pain, there are drawbacks associated with their chronic use, including tolerance, dependence, constipation, and addiction liability (Benyamin et al., 2008). The development of tolerance to the analgesic effects of opioids often leads to dose escalation, which may contribute to opioid misuse and abuse (Ballantyne and LaForge, 2007). Further, individuals who are dependent on opioids may misuse them to prevent withdrawal (Ross and Peselow, 2009, Bailey and Connor, 2005, Johnston et al., 2009, Ballantyne and LaForge, 2007). Opioid compounds that produce robust analgesia with limited development of tolerance and dependence could address a significant unmet medical need and provide an alternative to traditional opioid analgesics to prevent opioid misuse and abuse.

Opioids produce both their pain-relieving and adverse effects through stimulation of the mu opioid receptor (μ-receptor); therefore, creating more selective ligands for the μ-receptor is unlikely to reduce the incidence of adverse events. There are reports that the co-administration of μ-receptor agonist with a delta opioid receptor (δ-receptor) antagonist produces μ-receptor-mediated antinociception with reduced tolerance and dependence liabilities (Abdelhamid et al., 1991, Fundytus et al., 1995, Hepburn et al., 1997, Purington et al., 2009, Schiller, 2009, Anand et al., 2016, Váradi et al., 2016) and similar results have been found in δ-receptor knockout animals (Zhu et al., 1999). As a result the development of μ-receptor agonist/δ-receptor antagonist compounds – mixed efficacy ligands – has been explored and several peptide (Purington et al., 2011, Purington et al., 2009, Schiller et al., 1999, Schmidt et al., 1994, Anand et al., 2012, Cai et al., 2014), peptide-like (Balboni et al., 2002b, Balboni et al., 2002a, Salvadori et al., 1999, Lee et al., 2011, Bender et al., 2015, Mosberg et al., 2013), and alkaloid (Anathan et al., 1999, Anathan et al., 2004, Horan et al., 1993, Healy et al., 2013) compounds have been described. Noteworthy ligands are the peptides DIPPψNH_2 (Schiller et al., 1999) and VRP26 (Anand et al., 2016), the bivalent ligand MDAN-21 (Lenard et al., 2007), and the multifunctional opioid alkaloid UMB425 (Healy et al., 2013). All show some improvement over morphine, but both DIPPψNH_2

and UMB425 produce significant tolerance and dependence after chronic administration, and MDAN-21 was effective in some (Aceto et al., 2012, Daniels et al., 2005), but not all (Aceto et al., 2012), measures of antinociception. We previously reported that VRP26 produces no antinociceptive tolerance or physical dependence after seven day continuous administration and produces little conditioned place preference (Anand et al., 2016); however, VRP26 is difficult to synthesize and purify and therefore makes a poor drug candidate. While these μ-receptor agonist/δ-receptor antagonist compounds display promising effects *in vivo*, there is still room for improvement.

We have previously described a series of peptidomimetics which display μ-receptor agonist/δ-receptor antagonist characteristics *in vitro* and produce opioid-mediated antinociception *in vivo* after peripheral administration (Bender et al., 2015, Harland et al., 2015, Mosberg et al., 2013). In this report, we evaluated the acute and chronic effects of these compounds after repeated escalating doses for five days and the role of δ-receptor in the development of μ-receptor-mediated tolerance and dependence.

METHODS

In Vitro Characterization of Compounds:

Cell Lines and Membrane Preparations: C6-rat glioma cells stably transfected with a rat μ (C6-μ-receptor) or rat δ (C6- δ-receptor) opioid receptor (Lee et al., 1999) and Chinese hamster ovary (CHO) cells stably expressing a human κ (CHO-κR) opioid receptor (Husbands et al., 2005) were used for all *in vitro* assays. Cells were cultured and membranes prepared as previously described (Anand et al., 2012).

Radioligand Binding Assays: Radioligand binding assays were performed as previously described (Anand et al., 2012). In brief, assays were performed using competitive displacement of 0.2 nM [^{3}H]diprenorphine (250 μCi, 1.85TBq/mmol) by the test compound from membrane

preparations containing opioid receptors. The assay mixture, containing membranes (approximately 20 μg protein/tube) in 50 mM Tris-HCl buffer (pH 7.4), [^{3}H]diprenorphine, and various concentrations of test compound, was incubated on a shaker at room temperature for 1 h to allow binding to reach equilibrium. The samples were filtered through Whatman GF/C filters and washed five times with 50 mM Tris-HCl buffer (pH 7.4). The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail in a Wallac 1450 MicroBeta (Perkin-Elmer, Waltham MA, USA). Nonspecific binding was determined using 10 μM naloxone. The results presented are the mean ± SEM from three individual assays performed on three different days. Each individual assay is performed in duplicate and then averaged.

Radioligand Binding Assays in Sodium. Binding assays were performed by competitive displacement of [^{3}H]diprenorphine (250 μCi, 1.85 TBq/mmol) by test compound. The assay mixture contained the following components: assay buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4), various concentrations of test compound diluted in buffer, 0.2 nM [^{3}H]diprenorphine and membrane preparations containing opioid receptors (approximately 20 μg protein/tube) supplemented with 10 μM GTPγS. Nonspecific binding was determined using 10 μM naloxone. The assay plate was incubated at room temperature on a shaker for 75 min to allow binding to reach equilibrium. The mixture was then vacuum filtered through Whatman GF/ C filters using a Brandel harvester (Brandel; Gaithersburg, MD, USA) and washed 5 times with 50 mM Tris-HCl buffer. Retained radioactivity was measured as described above. The results presented are the mean ± SEM from three individual assays performed on three different days. Each individual assay is performed in duplicate and then averaged.

Stimulation of GTPγ[^{35}S] Binding: Agonist stimulation of [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate ([^{35}S]GTPγS, 1250 Ci, 46.2TBq/mmol) binding was measured as described previously (Anand et al., 2012). Briefly, membranes (10-20 μg of protein/tube) were incubated 1 h at room temperature in GTPγS buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4)

containing 0.1 nM [^{35}S]GTPγS, 30 μM guanosine diphosphate **(**GDP), and varying concentrations of test compound. Test compound stimulation of [^{35}S]GTPγS was compared with 10 μM standard compounds [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) at μ-receptor and D-Pen^{2,5}- enkephalin (DPDPE) at δ-receptor. The reaction was terminated by rapidly filtering through GF/C filters and washing ten times with GTPγS buffer and retained radioactivity was measured as described above. The results presented are the mean ± SEM from three individual assays performed on three different days. Each individual assay is performed in duplicate and then averaged; maximal stimulation was determined using nonlinear regression analysis to fit a logistic equation to the competition binding.

Determination of K_e: Agonist stimulation of [^{35}S]GTPγS binding by the known standard agonist DPDPE at δ-receptor was measured as described above. This was then compared to [^{35}S]GTPγS binding stimulated by DPDPE in the presence of test compound. Both conditions produced 100% stimulation relative to DPDPE. The difference between the EC_{50} of DPDPE alone and in the presence of test antagonist is the shift in dose response. The K_e was then calculated as K_e = (concentration of compound)/ (Dose response shift – 1). The results presented are the mean ± SEM from three individual assays performed on three different days. Each individual assay is performed in duplicate and then averaged; maximal stimulation was determined using nonlinear regression analysis to fit a logistic equation to the competition binding data.

Calculation of relative efficacy at μ-receptor. Agonist efficacy was calculated based on the ability to stimulate [^{35}S]GTPγS according to the equation: efficacy = $0.5\times(E_{max,test}/E_{max,std})\times(1+(K_{itest}/EC_{50test}))$, where $E_{max,test}$ is the maximum stimulation by test agonist, $E_{max,std}$ is the maximum stimulation by DAMGO, K_{itest} is the affinity of test agonist in buffer containing sodium, and EC_{50test} is the potency of test agonist. Hill slopes for all of the binding and functional data were not significantly different from one, allowing use of the Ehlert equation (Quock et al., 1999).

Data Normalization. Data for all *in vitro* competition binding assays are normalized such that basal (in the presence of 10 μM naloxone) and total binding (in the absence of any drug) are set

to zero and 100 percent binding respectively. Data for all *in vitro* [35S]GTPgS assays are normalized such that basal (in the absence of drug) and total (in the presence of 10 μM standard agonist) are set to zero and 100 percent stimulation respectively. This normalization is used to account for variation between membrane preparations or assays.

In Vivo Characterization of Compounds:

Drug preparation. All compounds were administered by intraperitoneal (*ip)* or subcutaneous (*sc)* injection in a volume of 10 mL/kg of body weight. Morphine sulfate, **AMB47 trifluroacetic acid salt (TFA)**, **AMB46 TFA**, and naltrexone HCl (NTX; Tocris, Biosciences, Minneapolis, MN, USA) were dissolved in sterile saline (0.9% NaCl *w/v*). **AAH8 TFA** was dissolved in 10:10:80 ethanol:Alkamuls 620 (Solvay, St. Louis, MO, USA):sterile water. SNC80 was dissolved in 1 M HCl and brought to a final concentration of 3% HCl (*v/v)* with sterile water. Naltrindole HCl (NTI; Tocris, Biosciences, Minneapolis, MN, USA) was prepared in sterile water.

Animals. Male and female C57BL/6 δ-receptor knockout mice (B6.129S2-*Oprd1*^{tm1Kff}/J stock number 007557, Jackson Laboratory, Sacramento CA, USA) and their wildtype littermates, C57BL/6 μ-receptor knock out mice (B6.129S2-*Oprm1*^{tm1Kff}/J stock number 007559, Jackson Laboratory, Sacramento CA, USA), or C57BL/6 wildtype mice (Stock number 000664, Jackson Laboratory, Sacramento CA, USA) weighing between 20-30g at 8-16 weeks old, were used for behavioral experiments. Knockout animals were bred in-house from heterozygous breeding pairs or trios. Mice were group-housed with a maximum of 5 animals per cage in clear polypropylene cages with corn cob bedding and nestlets as enrichment. Mice had free access to food and water at all times, except during measurements of fecal boli production. Animals were housed in pathogen free rooms maintained between 68-79°F and humidity between 30-70% humidity with a 12h light/dark cycle with lights on at 7:00 AM. Experiments were conducted in the housing room during the light cycle. Each mouse was used in only one experiment either for acute antinociception, tolerance, physical dependence, tail suspension test (TST), conditioned place

preference, or measurement of constipating effects. C57BL/6 mice are the background strain for all the genetic knockout strains used in this study. C57BL/6 mice were used for all studies as this species is commonly used in pharmacological and behavioral research. Studies were performed in accordance with US National Research Council's Guide for the Care and Use of Laboratory Animals (Council, 2011) and the ARRIVE guidelines (Kilkenny et al., 2010). For antinociception and constipation assays, experiments were not blinded to drug conditions due to the complication of multiple drug doses required for escalating doses. However, there were limited *a priori* expectations for drug effects as most compounds tested are novel entities.

Antinociception. Antinociceptive effects were evaluated in the mouse WWTW assay. Withdrawal latencies were determined by briefly placing a mouse into a cylindrical plastic restrainer and immersing 2-3 cm of the tail tip into a water bath maintained at either 50°C or 55°C. The latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cutoff time of 20 sec (50°C) or 15 sec (55°C) to prevent tissue damage; baseline latencies were consistent for each assay: 3-5 sec for 50°C and 2-3 sec for 55°C.

Acute antinociceptive effects were determined using a cumulative dosing procedure (n=6 animals per treatment group). Each mouse received an injection of saline *ip* and then 30 min later baseline withdrawal latencies were recorded, mice were then given an *ip* injection of either saline or 1 mg kg^{-1} naltrexone (NTX) and withdrawal latencies recorded 30 min later. Following baseline determinations, cumulative doses of the test compound were given *ip* at 30 min intervals. Thirty min after each injection, the tail withdrawal latency was measured as described above.

For antinociceptive tests, agonist-stimulated antinociception is expressed as a percentage of maximum possible effect (% MPE), where % MPE = (post-drug latency−baseline latency) ÷ (cutoff latency − baseline latency) × 100. Data are normalized to illustrate the difference in ED_{50} values as basal and maximal values vary based on temperature. Experiments were run by two separate individuals across multiple days.

Tolerance experimental design. Antinociceptive dose effect curves for **AAH8** (n=12), **AMB46** (n=12), **AMB47** (n=12) and morphine (n=12) were established on the morning of Day 1 in wildtype C57BL/6 mice using the 50°C WWTW assay described above. Each group was then randomly divided such that 6 mice were assigned to receive repeated drug injections and 6 mice were assigned to receive repeated saline injections.

On the morning of day 1 a dose response curve for the test compound was established up to 10 mg kg^{-1} *ip,* and mice were then given an injection of 10 mg kg^{-1} test compound *ip* at 7 pm on the evening of day 1. For the remainder of the experiment mice were given twice daily injections at 7 am and 7 pm; an escalating drug regimen was used such that mice received 20 mg kg^{-1} test compound twice daily (BID) on day 2, 30 mg kg^{-1} test compound BID on day 3, 40 mg kg^{-1} test compound BID on day 4, and 50 mg kg^{-1} test compound BID on day 5. Cumulative dose/effect curves were established for all mice on the morning of day 6 for their respective test compounds. Data are presented as mean ± SEM for each treatment group before and after repeated treatment.

To determine agonist potency before and after repeated treatment with drug or vehicle, dose-response curves and ED_{50} values were calculated for each mouse and then averaged within each chronic treatment group. To calculate ED_{50} values for each mouse in the warm water tail withdrawal assay, the 50% level of maximum effect was determined from a linear regression analysis of individual latency to tail flick data, using only the linear portion of the curve and including only one dose that produced <10% of the baseline latency and one dose that produced >90% of the maximum latency (Jutkiewicz et al., 2011). ED_{50} values from each mouse were then averaged for each treatment group (mean ± SEM). Statistical comparisons in ED_{50} values were made using a repeated measures, factorial ANOVA for each compound.

In a separate experiment antinociceptive dose response curves for morphine were established using the mouse WWTW assay in δ-receptor knockout mice (n=6) and their wildtype littermates (n=6). Mice were given twice daily escalating doses of morphine and dose response curves pre-

and post-escalating doses were performed as described above. Experiments were run by two separate individuals across multiple sessions.

Physical dependence experimental design. Wildtype C57BL/6 (n=6) or δ-receptor KO (n=6) mice were treated for 5 days with either saline or escalating doses of test compound twice daily as described above. On the morning of day 6, mice were given 50 mg kg^{-1} test compound, morphine, or saline *ip*, then returned to their home cages. Two hours later, mice were given 10 mg kg^{-1} naltrexone *ip* and placed individually in clear plastic observation cages (10 in X 6 in X 8 in) without bedding. Mice were observed for jumps as a sign of opioid withdrawal for 30 min after naltrexone injection. Statistical comparisons of the number of jumps recorded were assessed using a one way ANOVA. Experiments were run by two separate individuals across multiple sessions.

Tail Suspension Test (TST). Mice were pretreated with either vehicle (n=6), 3.2 mg kg^{-1} naltrindole (NTI; n=6), a single dose (1-10 mg kg^{-1}) of test compound (n=6 per dose), or 10 mg kg^{-1} morphine (n=6) *sc* either 30 min prior to injection of 3.2 mg kg^{-1} SNC80 or vehicle *sc*. Thirty minutes after SNC80 (or vehicle) injection, mice were suspended by their tail from a height of ~40 cm using tape for 6 minutes and behavior was recorded using a Sony HDR-CX220 digital camcorder. Videos were scored by observers blind to the test condition and the total time mice spent immobile was summed for each animal and then averaged within each treatment group. Immobility was defined as the animal remaining motionless or making only minor, non-escape related movements. Statistical comparisons in immobility were made using a two way ANOVA. TST videos were scored by a separate individual who did not run the assay and was blinded to experimental conditions.

Conditioned place preference and locomotor activity.

Apparatus. A two compartment place conditioning apparatus (MedAssociates, Inc. St. Albans, VT) was used for all conditioned place preference (CPP) studies. The compartmentalized box was divided into two equal size sections (8 in x 5 in x 5 in), accessed through a single, manual,

guillotine door. The compartments differed in the wall color and floor texture (black walls with rod flooring vs. white walls with mesh flooring). Time spent in each chamber, number of beam breaks (used as a measure of locomotor activity), and number of entrances to each side were recorded using infrared photobeam detectors.

Conditioned place preference protocol. Experiments consisted of three phases: bias evaluation (2 days), conditioning (5 days), and testing (1 day).

Bias evaluation of CPP. Wildtype mice were placed randomly into one chamber on day 1 and the opposite chamber on day 2 and then allowed to freely explore the apparatus for 30 min. If mice exhibited a greater than 70% pre-conditioning compartment bias they were discarded from the study; no mice were discarded based on this criterion.

Conditioning phase of CPP. Mice were randomly assigned to be conditioned with either 10 mg kg^{-1} **AAH8** (n=6), 10 mg kg^{-1} morphine (n=6) or saline (n=6) in either the black or white chamber. During conditioning mice were given a saline injection (*ip*) and immediately placed in the saline-paired chamber for 30 min; 6 h later mice were given an injection of either **AAH8**, morphine, or saline (*ip*) and immediately placed in the drug-paired chamber for 30 min. During all conditioning sessions, movement and activity were recorded.

Test day of CPP. Test day was always performed the day after the final conditioning session. Mice were randomly placed in either compartment and allowed to roam freely for 30 min. No injection was given on test day. Time spent in each chamber, beam breaks, and entrances to each side were recorded. CPP scores were calculated as the difference between time spent on the drug-paired side on test day and the average of time spent on the future drug-paired side on the two bias evaluations.

Experiments were run by two individuals across multiple sessions.

Measurement of Fecal Bolus Production. Tinted food was prepared by combining 25g of regular chow with 40 mL of water and 0.25 mL of blue food dye. The food pellets were allowed to

soften (approximately 2 h) and were mixed so that the food coloring was evenly distributed through the food paste. Mice were given 24 h access to tinted chow 1 week prior to an experiment in order to habituate them to the novel food preparation and then returned to regular chow. For experiments mice were single housed in cages free of bedding and were food deprived overnight; mice had *ad lib* access to water for the duration of the experiment. In the morning of the experiment, mice were given free access to tinted chow for 1 h. The tinted food was then removed, the cages wiped down, and the mice were given an injection of either drug or vehicle *ip* and access to approximately 3 g of normal chow for the remainder of the experiment. The weight of both the normal chow and the tinted chow was recorded both before and after the experiment. The time to first tinted fecal bolus and the number of tinted fecal boli were recorded every hour for 6 h.

Experiments were run by two individuals across multiple sessions.

Data and Statistical Analysis

Data analysis was performed using GraphPad Prism version 6.02 (GraphPad, San Diego, CA) or SPSS v22 with Tukey post-hoc tests to correct for multiple comparisons. K_i and EC_{50} values were calculated using nonlinear regression analysis to fit a logistic equation to the competition binding data. ED_{50} values were calculated using GraphPad Prism version 6.02 by extrapolating the 50% maximum effect from the straight line analysis of the individual dose effect curves (Jutkiewicz et al., 2011) and then ED_{50} values were averaged within treatment group. For *in vivo* experiments, 6 mice per experimental condition (e.g., per drug and per genotype) were used. For statistical tests, post hoc tests were run only when F achieved $P<0.05$ (α level was set to 0.05). There was no exclusion of any data in any studies. Treatment conditions were randomized across cages of mice and across at least 3 independent experiments. For in vivo studies, power analysis (α=0.05; 1-β=0.9) revealed that for a calculated effect size of 1-3 (Cohen's d), depending on the experiment, that a sample size of 4-6 mice per experimental condition would be needed (G*Power 3.1.9.2, Faul *et al.*, 2007). The data and statistical analysis comply with the

recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Materials: All reagents and solvents were purchased from commercial sources and used without further purification. All chemicals and biochemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or Fisher Scientific (Hudson, NH, USA), unless otherwise noted. All tissue culture reagents were purchased from Gibco Life Sciences (Grand Island, NY, USA). Radioactive compounds were purchased from Perkin-Elmer (Waltham, MA, USA).

Synthesis. AAH8, AMB46 and **AMB47** using the route previously described (Bender et al., 2015, Harland et al., 2015).

Nomenclature of Targets and Ligands Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

RESULTS

In Vitro Results

Affinity. As previously reported, **AAH8** (Harland et al., 2015), **AMB47,** and **AMB46** (Bender et al., 2015) all display low nanomolar binding affinity for μ-receptor and δ-receptor when binding assays are conducted in Tris buffer. Morphine, a prototypical μ-receptor ligand, binds with low nanomolar affinity to μ-receptor, preferring μ-receptor 50-fold over δ-receptor (Table 1). As it has been demonstrated that sodium ions can alter the affinity of opioid ligands for their receptors (Simon and Groth, 1975, Pert et al., 1973, Selley et al., 2000), we assessed the affinity of **AAH8, AMB47, AMB46,** and morphine for μ-receptor and δ-receptor in the presence of sodium. Sodium ions decrease the affinity at μ-receptor and δ-receptor for all compounds tested, though the fold change in affinity is different for different compounds at each receptor (Table 1).

Efficacy. **AAH8, AMB47,** and **AMB46** are full agonists in the [^{35}S]GTPγS assay at the μ-receptor as compared with DAMGO, with low nanomolar EC_{50} values; morphine is a partial agonist as compared with DAMGO. The relative efficacy of these compounds *in vitro* is as follows: **AAH8>AMB46>AMB47>**morphine. **AAH8 AMB47,** and **AMB46** are antagonists at the δ-receptor as they attenuate DPDPE-stimulated [^{35}S]GTPγS binding, with antagonist potency values (K_e) values in the nanomolar range; in this assay, NTI, a known δ-receptor antagonist, displays a K_e value of 0.13 ± 0.03 nM. Morphine is a low-affinity, partial agonist at the δ-receptor and as such does not shift the dose response curve for DPDPE and does not produce a measurable K_e value *in vitro* (Table 1).

In Vivo Results

μ-Receptor-Mediated Acute Antinociceptive Effects. The antinociceptive effects of **AAH8, AMB47, AMB46,** and morphine were assessed using the 50ºC warm water tail withdrawal (WWTW) assay in wildtype C57BL6/J mice pretreated with either saline or 1 mg kg^{-1} naltrexone (NTX), a non-selective opioid antagonist, to determine if antinociceptive effects are opioid-mediated *in vivo*. Consistent with earlier results (Bender et al., 2015, Harland et al., 2015), all compounds produce maximal antinociceptive effects at 10 mg kg^{-1} after *ip* injection in mice pretreated with saline (Figure 1). Pretreatment with 1 mg kg^{-1} NTX *ip* produces an approximate 3-fold parallel rightward shift in the dose response curves for **AAH8, AMB47, AMB46,** and morphine (Table 2). All compounds were then tested in μ-receptor knockout mice, to determine if the antinociceptive effects were μ-receptor-mediated. Consistent with *in vitro* results, neither the test peptidomimetics nor morphine produced any antinociception in μ-receptor knockout mice (Figure 1); ethylketocyclazocine, a known κ-receptor agonist (used as a positive control) produced dose-dependent antinociception (Supplemental Figure 1).

While the peptidomimetics are equipotent in the 50°C WWTW assay, when tested at 55ºC, differences in ED_{50} between the compounds are observed even though they are all still fully effective (Figure 1; Table 2). A one way ANOVA of ED_{50} values ($F(3,40) = 398.9$) shows a

main effect of drug, demonstrating that **AAH8** and morphine are significantly more potent than either **AMB47** or **AMB46** ($F(3,40) = 41.8$).

In Vivo Acute δ-receptor Antagonist Effects. To investigate whether **AAH8, AMB47,** and **AMB46** function as centrally-active δ-receptor antagonists *in vivo*, we examined their ability to block the antidepressant-like effects of a δ-receptor agonist, SNC80, in the tail suspension test (TST) as compared with the prototypic δ-receptor antagonist naltrindole (NTI; Figure 2A). A two way ANOVA of the data shows a significant main effect of SNC80 dose ($F(1,30) = 101.1$) and of pretreatment (NTI, morphine or vehicle, $F(2,30) = 45.83$), and an interaction of pretreatment X SNC80 dose ($F(2,30) = 14.10$). Mice treated with 3.2 mg kg^{-1} SNC80 *(sc*) alone display a significant decrease in immobility as compared with vehicle treated mice. This SNC80-induced decrease in immobility is blocked by pretreatment with 3.2 mg kg^{-1} NTI (*sc)*; NTI pretreated mice have immobility scores that are not statistically different from immobility scores in vehicle-treated mice. Pretreatment with morphine produces small, though not statistically significant, decreases in immobility scores in vehicle-treated mice.

δ-receptor antagonist dose response curves were established for **AAH8, AMB47,** and **AMB46** as pretreatments to SNC80 (Figure 2B). One way ANOVAs were performed for each peptidomimetic dose response curve in the mouse TST comparing each dose to SNC80 alone and peptidomimetic alone (control conditions); all three one way ANOVAs showed a significant effect of treatment: **AAH8** $F(4, 25) = 12.88$, **AMB47** $F(5, 30) = 36.47$, and **AMB46** $F(5, 30) = 24.62$. The large dose of each peptidomimetic alone (10 mg kg^{-1}) produces immobility levels similar to that observed with no drug conditions (Figure 2A) and SNC80 alone significantly decreases immobility. Mice pretreated with the smallest doses tested of **AAH8** (1 mg kg^{-1}), **AMB47** (0.32 mg kg^{-1}), and **AMB46** (0.32 mg kg^{-1}) display immobility scores similar to those produced by SNC80 alone. However, pretreatment with larger doses of **AAH8** (3.2 and 10 mg kg^{-1}), **AMB47** (1, 3.2, and 10 mg kg^{-1}) and **AMB46** (1, 3.2, and 10 mg kg^{-1}) prior to SNC80 significantly attenuates SNC80-induced decreases in immobility, and these scores are not statistically different from treatment with peptidomimetic alone. IC_{50} values derived

peptidomimetic dose effect curves show that **AAH8**, **AMB47**, and **AMB46** have similar δ-receptor antagonist potencies *in vivo* (IC_{50} 2.06 mg kg^{-1}, 1.66 mg kg^{-1}, and 1.61 mg kg^{-1} respectively). NTI is reported to have an IC_{50} of 2 mg kg^{-1} in the mouse TST in male C57BL6 mice (Naidu et al., 2007).

Development of Tolerance to Antinociceptive Action.

AAH8. A factorial ANOVA of the **AAH8** dose effect curves before and after repeated treatment shows no interaction between factors (**AAH8** dose X day X repeated treatment). There is a main effect of **AAH8** dose ($F(4,40) = 510.28$), demonstrating that **AAH8** produces dose-dependent increases in antinociceptive effects, but there is no effect of day (day 1 vs day 6) or repeated treatment (saline vs **AAH8**). A separate two way, repeated measures ANOVA of the ED_{50} values only also demonstrates that there is no significant shift in the dose response curves for **AAH8** before and after repeated treatment in either saline or **AAH8** treated groups (day 1 saline treated group: 4.73 ± 0.002 mg kg^{-1}, day 1 **AAH8** treated group: 4.74 ± 0.02; day 6 saline treated group: 4.73 ± 0.002 mg kg^{-1}, day 6 **AAH8** treated group: 4.74 ± 0.0001; Figure 3A).

AMB47. Similar to **AAH8**, a factorial ANOVA of the **AMB47** dose effect curves before and after repeated treatment shows no interaction between factors (**AMB47** dose X day X repeated treatment). There is a main effect of **AMB47** dose ($F(4,40) = 1129.71$), demonstrating that **AMB47** produces dose-dependent increases in antinociceptive effects, but there is no effect of day (day 1 vs day 6) or repeated treatment (saline vs **AMB47**). A separate two way, repeated measures ANOVA of the ED_{50} values also demonstrates that there is no significant shift in the dose response curves for **AMB47** before and after repeated treatment in either saline or **AMB47** treated groups (day 1 saline treated group: 4.73 ± 0.19 mg kg^{-1}, day 1 AMB47 treated group: 4.64 ± 0.09**,** day 6 saline treated group: 4.95 ± 0.24 mg kg^{-1}, day 6 AMB47 treated group 4.73 ± 0.14; Figure 3B).

AMB46. A factorial ANOVA comparing **AMB46** dose effect curves before and after repeated treatment shows a significant interaction (**AMB46** dose X day X repeated treatment; $F(4,40) =$

23.245) and significant main effects of **AMB46** dose (F(4,40) = 1096.44), day (1 vs 6 F(1,10) = 12.71), and repeated treatment (saline vs **AMB46**, F(1,10) = 8.60). Repeated treatment with **AMB46**, but not saline, produces a 3-fold, rightward, parallel shift in the **AMB46** dose response curve (repeated treatment X day interaction F(1,10) = 51.71). After 5 days of treatment with escalating doses of **AMB46**, the ED_{50} of the **AMB46** dose effect curve is more than 3.5-fold larger on day 6 (17.04 ± 1.25 mg kg^{-1}) as compared with day 1 (4.63 ± 1.06 mg kg^{-1}). The **AMB46** dose effect curves in mice treated with saline are not different on day 1 and 6 (day 1: 5.18 ± 0.31 mg kg^{-1} vs day 6: 4.73 ± 0.006 mg kg^{-1}). A separate two way, repeated measures ANOVA of the ED_{50} values shows significant main effects of repeated treatment (F (1, 10) = 78.25) and day (F (1, 10) = 89.68)), and an interaction of repeated treatment X day (F (1, 10) = 103.8; Figure 3C).

Morphine. A factorial ANOVA comparing morphine dose effect curves before and after repeated treatment shows a significant interaction (morphine dose X day X repeated treatment; F(4,40) = 25.07) and significant main effects of morphine dose (F(4,40) = 1008.61), day (1 vs 6; F(1,10) = 51.62), and repeated treatment (saline vs morphine; F(1,0) = 35.71). Repeated morphine, but not repeated saline, treatment produces a 3-fold, rightward, parallel shift in the morphine dose response curve (treatment X day interaction F(1,10) = 31.79). After 5 days of treatment with escalating doses of morphine, the ED_{50} of the morphine dose effect curve is more than 3-fold larger on day 6 (14.72 ± 1.39 mg kg^{-1}) as compared with day 1 (4.74 ± 0.11 mg kg^{-1}, F(1,10) = 9.881). The morphine dose effect curves in mice treated with saline are not different on day 1 and 6 (day 1: 4.93 ± 0.32 mg kg^{-1} vs day 6: 4.53 ± 0.26 mg kg^{-1}). A separate two way, repeated measures ANOVA of ED_{50} values shows a significant effect of repeated treatment (F (1, 10) = 45.56) and day (F (1, 10) = 44.96)), and an interaction of chronic treatment X day (F (1, 10) = 52.83; Figure 3D).

δ-receptor knockout mice and their wildtype littermates.

A factorial ANOVA comparing morphine dose effect curves in δ-receptor knockout mice and their wildtype littermates before and after repeated morphine treatment shows a significant interaction (morphine dose X day X genotype; $F(4,40) = 32.89$) and significant main effects of morphine dose ($F(4,40) = 962.39$), day tested (1 vs 6; $F(1,10) = 4.14$), and genotype (δ-receptor knockout vs wildtype; $F(1,0) = 46.03$). Repeated morphine administration in wildtype mice, but not δ-receptor knockout mice, produces a 3-fold, rightward, parallel shift in the morphine dose response curve (genotype X day interaction $F(1,10) = 33.28$). After repeated treatment with escalating doses of morphine, the ED_{50} of the morphine dose effect curve in wildtype littermates is 3-fold larger on day 6 (14.73 ± 0.79 mg kg^{-1}) as compared with day 1 (4.63 ± 0.26 mg kg^{-1}). The morphine dose effect curves in δ-receptor knockout mice are not different on day 1 and 6 (day 1: 4.73 ± 0.13 mg kg^{-1} vs day 6: 4.74 ± 0.24 mg kg^{-1}; Figure 5A). A separate two way, repeated measures ANOVA of ED_{50} values shows a significant effect of genotype ($F(1, 10) = 196$) and day ($F(1, 10) = 97.9$)), and an interaction of chronic treatment X day ($F(1, 10) = 97.8$).

Physical Dependence.

In wildtype mice treated repeatedly with increasing doses of morphine, **AMB47,** or **AMB46** for five days, naltrexone precipitates jumping behavior **(**one way ANOVA ($F(4,25) = 8.15$; Figure 4). In morphine and **AMB46** treated mice, naltrexone elicits significantly more jumps than in mice treated with saline or **AAH8**. The number of naltrexone-precipitated jumping in **AMB47** treated mice is significantly larger than in saline treated mice, but not **AAH8** treated mice. There is no difference between mice treated chronically with saline or **AAH8.** After five days of escalating morphine doses, naltrexone precipitates a similar number of withdrawal jumps in δ-receptor knockout mice and wildtype littermates (Figure 5B).

Conditioned Place Preference. The rewarding effects of both morphine and **AAH8** were explored using the conditioned place preference (CPP) assay (Figure 6A; one way ANOVA ($F(2, 15) = 6.382$)). Conditioning with morphine produces a significant increase in time spent on the morphine-paired side of the apparatus as compared with conditioning with saline or **AAH8**

(Figure 6A). Conditioning with **AAH8** does not increase time spent on the **AAH8**-paired side of the apparatus as compared to saline conditioning.

Locomotor activity was recorded during all conditioning sessions. A two way, repeated measures ANOVA shows a significant main effect of drug ($F(2,15) = 12.10$), but no effect of day and no significant interaction. Morphine produces a significant increase in locomotor activity as compared with saline on both day 1 and day 5. **AAH8** does not increase locomotor activity on either day 1 or day 5 (Figure 6B).

Production of Fecal Boli. Mice treated with saline produce significantly more tinted fecal boli than those treated with **AAH8, AMB47, AMB46,** or morphine (one way ANOVA $F(4,27) = 30.77$; Figure 7), and there was no difference in number of tinted fecal boli between mice treated with **AAH8, AMB47, AMB46,** and morphine. A one way ANOVA shows that mice treated with saline produced tinted fecal boli significantly earlier than those treated with **AAH8, AMB47, AMB46,** or morphine (one way ANOVA $F(4,35) = 49.14$; Supplementary Figure 1). The time to first tinted fecal bolus is not statistically different in mice treated with **AAH8, AMB47, AMB46,** and morphine (Supplementary Figure 1).

DISCUSSION and CONCLUSIONS

The data described in this report demonstrate that the structurally-related, mixed efficacy opioid ligands **AAH8**, **AMB47** and **AMB46** produce similar effects *in vivo* after acute administration but have different profiles of activity following repeated administration. Consistent with their *in vitro* profile, these compounds act as μ-receptor agonists and δ-receptor antagonists *in vivo* (Figures 1 and 2). They produce dose-dependent antinociceptive effects with similar potencies and are fully effective, as compared with morphine, in the 50ºC WWTW assay. Naltrexone attenuates the antinociceptive effects to a similar extent as shown by equivalent shifts in their ED_{50}s (Table 2), suggesting that these antinociceptive effects are opioid receptor-mediated. Further, the antinociceptive effects of these peptidomimetics are completely attenuated

in μ-receptor knockout mice, demonstrating that the antinociception is μ-receptor-mediated (Figure 1). Doses that are fully effective in a 50ºC WWTW assay also decrease the production of fecal boli produced over a 6 hour window (Figure 7), consistent with the effects of morphine. As δ-receptor antagonists, these compounds attenuate SNC80-induced decreases in immobility in the mouse TST (Figure 2) with IC_{50}s similar to NTI (Naidu et al., 2007). These compounds may be slightly less effective than the known δ-receptor antagonist NTI, which could be due to their μ-receptor agonist activity, consistent with the small, non-significant decreases in immobility produced by morphine alone (Figure 2). Overall, these data demonstrate that **AAH8**, **AMB47**, and **AMB46** simultaneously function as μ-receptor agonists and δ-receptor antagonists *in vivo*. Further, the δ-receptor antagonist properties of these compounds do not alter their acute antinociceptive or constipating effects.

The acute behavioral effects of these compounds are consistent with their *in vitro* profile as μ-receptor agonists. All three peptidomimetics display high affinity μ-receptor binding affinities in the absence of sodium. In the presence of sodium ions, the affinity of these ligands for μ-receptor is decreased, as expected since sodium ions stabilize inactive receptor states and alter agonist affinity (Pert et al., 1973, Selley et al., 2000, Simon and Groth, 1975); however, these compounds still have K_i values in the nanomolar range and demonstrate higher μ-receptor affinity than morphine under these conditions. In addition, these ligands are more efficacious than morphine *in vitro*. Consistent with this idea, their calculated relative efficacies using the Ehlert equation (Quock et al., 1999) can be rank ordered: **AAH8**>**AMB46**>**AMB47**≈morphine. However, these *in vitro* data do not effectively predict their potency and efficacy *in vivo*. For example, in a 50°C WWTW assay, these compounds demonstrate similar potency to morphine and produce a maximal response at similar doses, but under higher efficacy-requiring conditions (55°C WWTW), the dose effect curves for **AMB46** and **AMB47** are shifted to a greater extent than **AAH8** and morphine. These findings would suggest that **AAH8** and morphine are higher efficacy agonists than **AMB46** and **AMB47**, which is not entirely consistent with their *in vitro* profile. Suggesting, some unidentified pharmacokinetic parameter may be responsible for the

differences between these ligands *in vivo*. It is possible that differential plasma protein binding, metabolism, or distribution to the active site, presumably the CNS, leads to different local concentrations of peptidomimetic, which may explain the discrepancies between *in vitro* and *in vivo* potencies and efficacies. Future work will explore how the pharmacokinetic properties of compounds in this series alters their acute and chronic effects.

While these compounds are μ-receptor agonists *in vitro*, they do not stimulate δ-receptor-mediated [^{35}S]GTPγS binding in cells and attenuate δ-receptor agonist-stimulated G protein activation, suggesting they are δ-receptor antagonists. Notably, these ligands differ in their affinity for δ-receptor *in vitro* over an 80-fold range. In the absence of sodium, these ligands have low nanomolar or sub-nanomolar affinity for δ-receptor with a rank order of **AAH8**>**AMB47**>**AMB46**. In the presence of sodium, the rank order for affinity at δ-receptor does not change, but the K_i values shift, inconsistent with neutral antagonist activity. These findings suggest that these compounds could potentially be low efficacy δ-receptor agonists (below the threshold for this assay). Again, these *in vitro* data do not correlate well with *in vivo* δ-receptor antagonist activity, since the three peptidomimetics display similar δ-receptor antagonist-like activity *in vivo* with equivalent potency (Figure 2).

Although these compounds have similar μ-receptor and δ-receptor activity following acute administration *in vivo*, their behavioral effects differ following repeated administration. For example, tolerance, as demonstrated by rightward shifts in the dose effect curves, is observed following repeated administration of morphine, **AMB46**, but not **AAH8** and **AMB47** (Figure 3). Naltrexone precipitates withdrawal in mice treated with repeated morphine, **AMB46**, and **AMB47**, but significantly fewer signs of withdrawal are observed in mice that receive repeated **AAH8**. Considering the *in vivo* effects of these 3 compounds evaluated in the current study, the rank order of most favorable profile is: **AAH8**>**AMB47**>**AMB46**≈morphine. Overall, the compound with the most promising profile is **AAH8** since it produces less tolerance and physical dependence as compared with morphine under the same conditions. In addition, **AAH8** also failed to produce conditioned place preference at a dose that produces significant antinociception

(Figure 6). These findings suggest that **AAH8** is less rewarding than morphine and, therefore, may be a safer analgesic than traditional opioids.

While this study identifies a promising candidate, it also highlights that the combination of a μ-receptor agonist with δ-receptor antagonist is not sufficient to prevent tolerance development since all of these compounds were δ-receptor antagonists *in vivo*. To further probe the disparities between these compounds in terms of tolerance development, we considered whether differences in 1) μ-receptor efficacy, 2) δ-receptor affinity and/or 3) μ-receptor:δ-receptor affinity ratio would correlate with the rank order of favorable profiles (**AAH8**>**AMB47**>**AMB46**≈morphine). In terms of μ-receptor efficacy, we hypothesized that high efficacy μ-receptor agonists would be less likely to produce tolerance due to a larger receptor reserve. *In vitro* relative efficacy calculations at μ-receptor orders the compounds: **AAH8**>**AMB46**>**AMB47**≈morphine, but *in vivo* we observe a different organization under the higher efficacy conditions such that **AAH8**=morphine>**AMB47**=**AMB46**. Therefore, *in vitro* relative efficacy does not appear to predict *in vivo* efficacy requirement, and compound efficacy *in vitro* or *in vivo* does not correlate with the lack of tolerance development.

While δ-receptor antagonist activity alone is not sufficient to prevent tolerance, it is likely that δ-receptor plays a significant role, as demonstrated by less tolerance development in mice δ-receptors. Some compound properties that do correlate with the lack of tolerance development under these conditions include: 1) δ-receptor affinity in both binding assay conditions or as determined K_e values and 2) μ-receptor:δ-receptor affinity ratios, such that high affinity binding at δ-receptor may protect against tolerance, and possibly, dependence. However, δ-receptor expression and/or signaling may be less relevant to the mechanisms involved in physical dependence, since precipitated withdrawal is similar in wildtype and δ-receptor knockout mice. Future studies will probe the role of δ-receptor in the effects of chronic administration of mixed efficacy opioid ligands. Furthermore, a single characteristic alone may not account for the lack of tolerance development with some of these ligands, but multiple features may be required to produce some preferred pharmacological profile, such as a combination of high efficacy μ-

receptor agonist activity and high affinity binding to δ-receptor. Still other mechanisms, not considered here, may be important in preventing tolerance development. For instance, activity at the κ-receptor may play an important role; all three peptidomimetics bind the κ-receptor with nanomolar affinity and both **AMB47** and **AMB46** display some κ-receptor activation in the GTPγS assay. It is possible that chronic activation of κ-receptor may play a role in the development of adverse effects associated with opioid use. Another possible factor to consider is that these peptidomimetics may activate distinct intracellular signaling pathways and may exhibit biased signaling at one or more of the opioid receptors. It has been proposed that developing biased μ-receptor agonists that favor G protein signaling over arrestin3 signaling might provide pain relief without the development of adverse effects (Kelly, 2013, Raehal et al., 2011). However, the loss of arrestin3 does not attenuate the development of adverse effects for all opioid agonists, suggesting that agonists produce adverse effect through different mechanisms or that other factors mediate adverse effects (Raehal and Bohn, 2011). Further, the theory is not supported by studies of G protein biased μ-receptor agonist TRV130 (Altarifi et al., 2017). Future work will explore these possibilities to determine what role, if any, they play development of adverse effects to opioid analgesics.

Overall, this report identifies a promising opioid ligand that produces antinociception without development of tolerance or dependence under the conditions tested; further, our lead compound, **AAH8,** is less rewarding than morphine. However, it also highlights that the combination of a μ-receptor agonist with a δ-receptor antagonist is not sufficient to prevent the development of tolerance or physical dependence since all of these compounds were δ-receptor antagonists *in vivo*. Future studies will test **AAH8** over longer periods of administration and in chronic pain models. Finally, we will continue to probe the mechanisms by which δ-receptor antagonist activity modifies tolerance development to μ-receptor agonists in order to better understand how these mixed efficacy ligands differ in their *in vivo* effects following repeated administration.

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Table and Figure Legends:

Table 1: Binding affinities (K_i) were obtained by competitive displacement of radiolabeled [^{3}H] diprenorphine in the presence or absence of sodium chloride. Efficacy data were obtained using [^{35}S]GTPγS binding assay. Efficacy is represented as percent maximal stimulation relative to standard agonists DAMGO (μ), DPDPE (δ) or U69,593 (κ) at 10μM concentrations. Relative efficacy at μ was calculated using the Ehlert equation. K_e values at δ were determined by shifting the dose response curve for DPDPE, a standard δ agonist. All values are expressed as mean ± SEM of three separate assays performed in duplicate. nd = not determined, dns = does not stimulate. n=3 for all experiments. a – data previously published in Harland et al., 2015; b – data previously published in Bender et al., 2015

Table 2: ED_{50} values for peptidomimetics and morphine tested in the WWTW assay with either a saline (50° and 55°C) or 1 mg kg^{-1} naltrexone (NTX; 50°C) pretreatment. ED_{50} values were calculated using a linear regression fit for the cumulative dose-response data from each individual mouse then averaged to get an ED_{50} value for each treatment group (n=6).

Figure 1: Cumulative dose response curves for (A) **AAH8**, (B) **AMB47** (C) **AMB46**, and (D) morphine in the mouse warm water tail withdrawal assay at 50°C (open symbols) or 55°C (closed symbols) in wildtype mice or at 50ºC in μ-receptor knockout mice (grey symbols). Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 2: **(A)** Immobility scores in the mouse TST for animals pretreated with either vehicle, 3.2 mg kg^{-1} NTI, or 10 mg kg^{-1} morphine 30 min before 3.2 mg kg^{-1} SNC80. Pretreatment with NTI attenuates SNC80-induced antidepressant-like effects, as expected for a δ antagonist. Morphine does not alter SNC80s effects in the TST. # indicates significance relative to vehicle

pretreatment **(B)** Dose response curves for **AAH8**, **AMB47**, and **AMB46** in the mouse TST. * indicates that all peptidomimetics are significantly different from SNC80 alone, # indicates **AMB47** and **AMB46** are significantly different from SNC80 alone, & indicates that all peptidomimetics are significantly different from 10 mg kg^{-1} peptidomimetic alone, $ indicates that **AMB47** and **AMB46** are significantly different from 10 mg kg^{-1} peptidomimetic alone. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 3: 5 days of chronic escalating treatment with **AAH8** (A) or **AMB47** (B) (10-50 mg kg^{-1} ip, twice daily) treatment *ip* (n=6) produces no shift in the dose effect curve in wild type BL6 mice. 5 days of chronic escalating morphine (D**)** or **AMB46 (C)** (10-50 mg kg^{-1} ip, twice daily) treatment *ip* (n=6), but not saline (n=6) produces a significant 3-fold rightward shift in the dose effect curve in wild type BL6 mice. * indicates significant difference relative to data from day 1. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 4: Wildtype mice were treated for 5 days with either saline or escalating doses of **AAH8, AMB47, AMB46,** or morphine (10-50 mg kg^{-1} ip, twice daily). Withdrawal was precipitated with 10 mg kg^{-1} NTX ip and number of jumps were counted. Animals treated chronically with **AMB47**, **AMB46**, and morphine experienced more NTX precipitated withdrawal jumps than animals treated chronically with saline or **AAH8**. *indicates significant difference relative to saline, # indicates significant difference relative to **AAH8**. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 5: (A) 5 days of chronic escalating treatment with morphine (10-50 mg kg^{-1} ip, twice daily) in δ KO mice (-/-) and their wild type littermates (+/+) produces no shift in the dose effect curve in δ KO mice, but produces a 3 fold rightward shift in wild type mice. (B) Wild type (+/+)

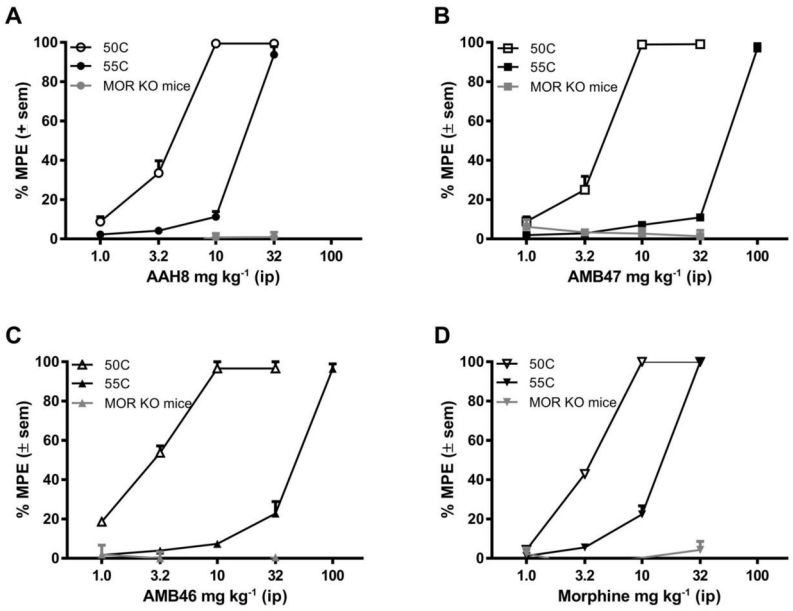
and δ KO (-/-) mice were treated for 5 days with escalating doses of morphine (10-50 mg kg^{-1} ip, twice daily). Withdrawal was precipitated with 10 mg/kg NTX. There was no significant difference in the number of jumps observed across genotype. * indicates a significant difference relative to data from day 1. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 6: Conditioned place preference (CPP) scores for animals trained for 5 days on either 10 mg kg^{-1} morphine, 10 mg kg^{-1} **AAH8** or saline for 5 days *ip*. CPP scores are defined as the difference between time spent on drug paired side pre- and post- conditioning measured in seconds. (A) Animals conditioned with morphine spent more time on the drug paired side of the CPP apparatus than those trained to either **AAH8** or saline. (B) Locomotor activity over 30 mins for 10 mg kg^{-1} morphine, 10 mg kg^{-1} **AAH8** and saline on Day 1 and Day 5. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Figure 7: Acute treatment with 10 mg kg^{-1} **AAH8, AMB47, AMB46,** and morphine all significantly reduce the number of fecal boli produced over 6 h as compared to saline controls. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Supplemental Figure 1. Cumulative dose response curves for morphine or the κ-receptor agonist ethylketocyclazocine (EKC) (or repeated saline injection) in the 50ºC mouse warm water tail withdrawal assay at 50°C in μ-receptor knockout mice. Data are plotted as mean ± SEM for all groups (n=6 for each group).

Supplemental Figure 2: Acute treatment with 10 mg kg^{-1} **AAH8, AMB47, AMB46,** and morphine all significantly increase the time to first tinted fecal bolus as compared to saline controls. Data are plotted as mean ± SEM for all groups (n=6 for each group).



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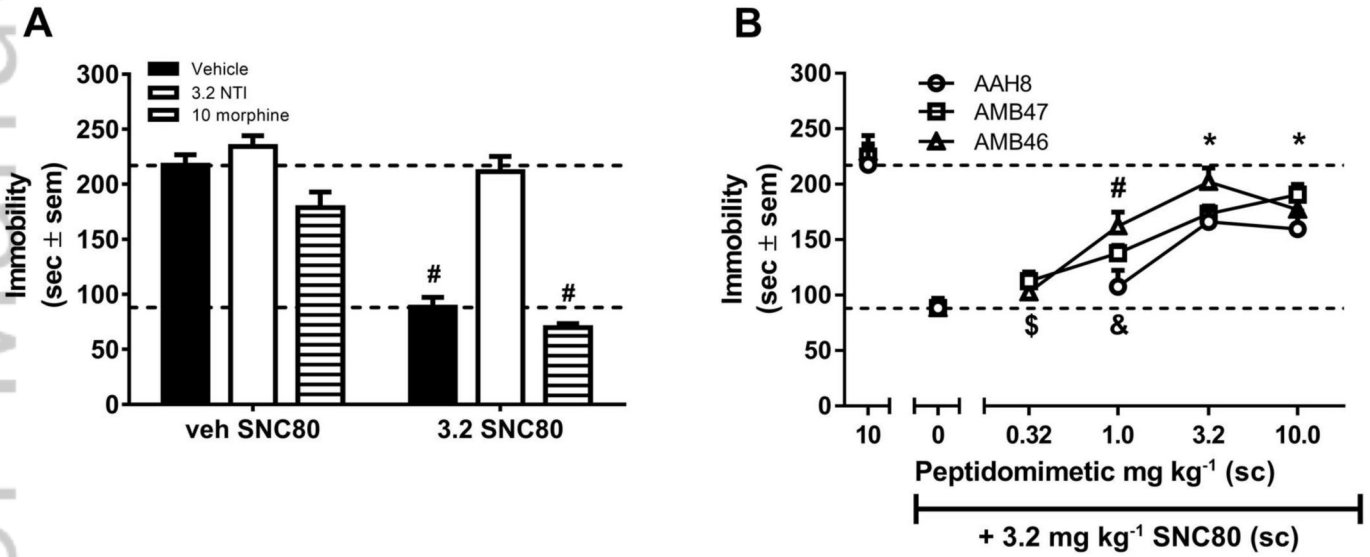

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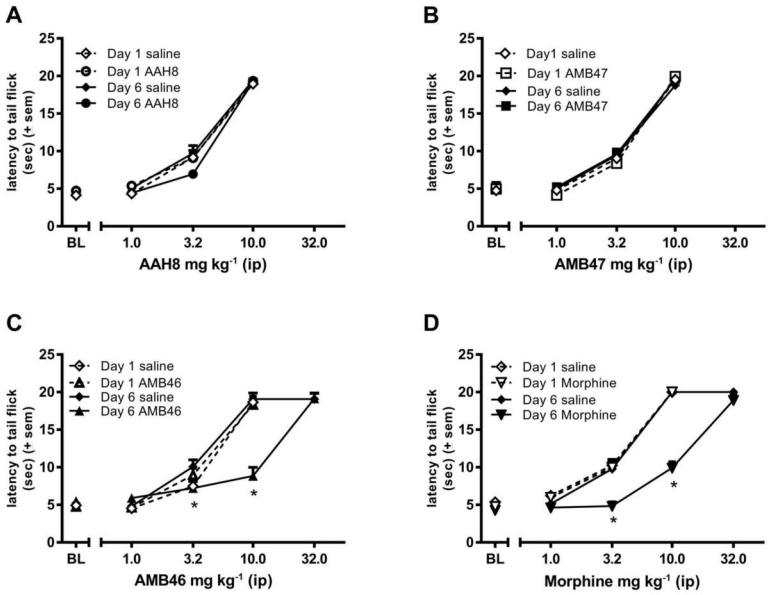

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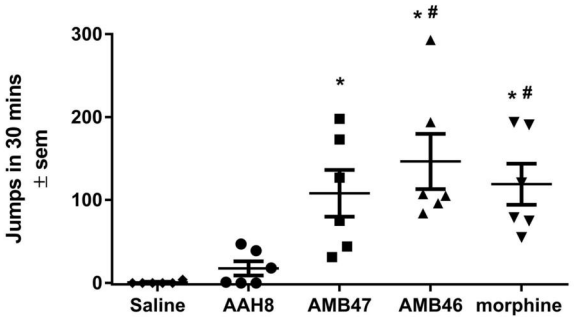

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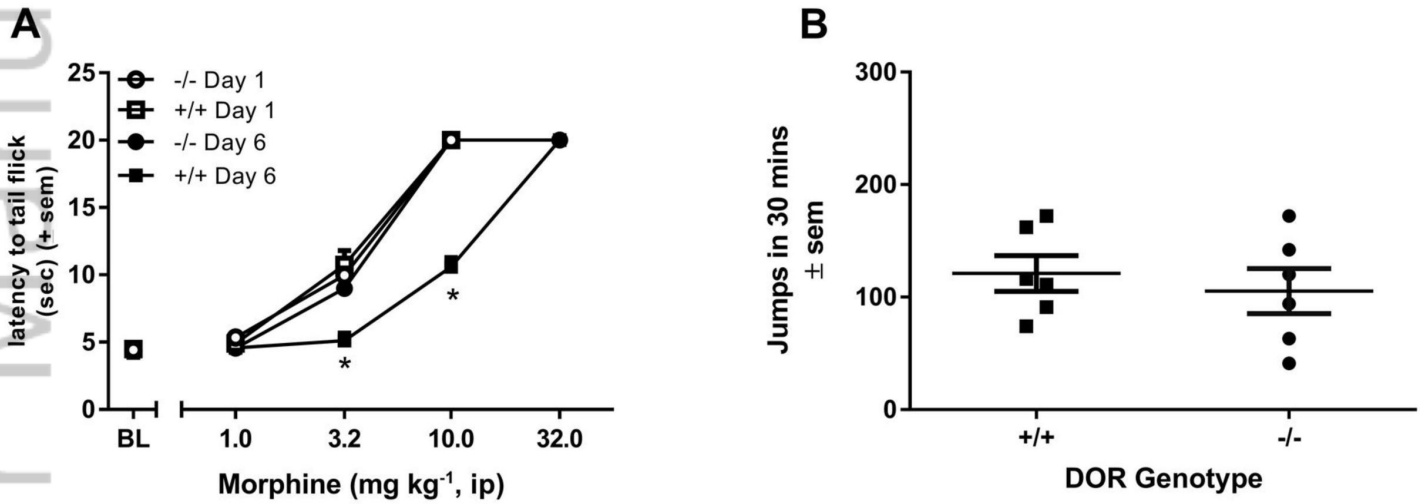

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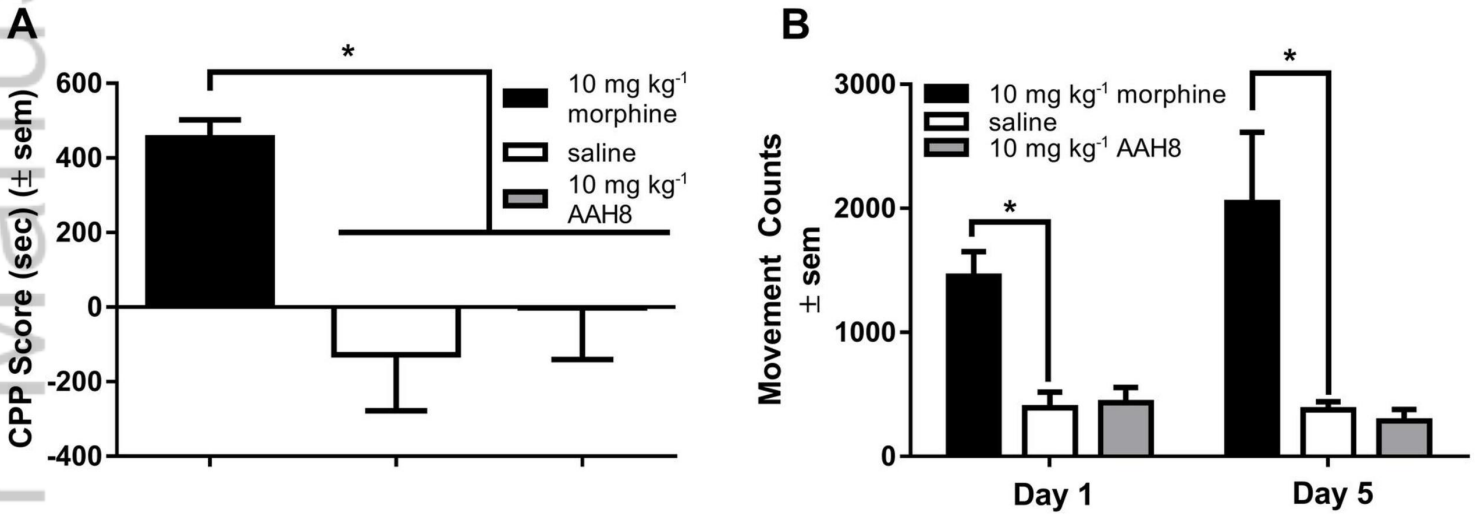

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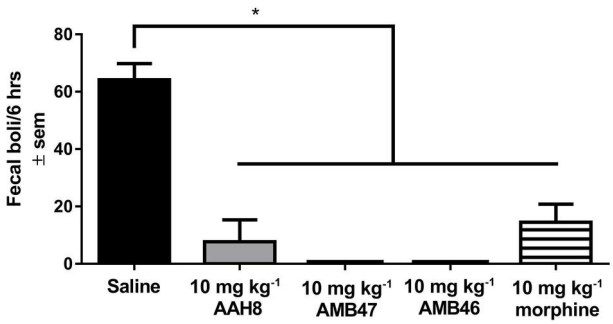

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List of Hyperlinks for Crosschecking

δ-receptor
http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=317

μ-receptor
http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=319

SNC80
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1611

Morphine
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1627

[^{3}H]diprenorphine
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1612

GTPγS
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=4207

DAMGO
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1647

DPDPE
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Naloxone
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1638

Naltrexone
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1639

Naltrindole
http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1641

Table 1

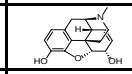
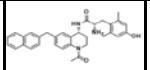
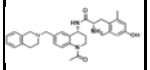
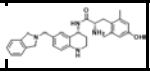
Cmpd	Structure	Ki in Tris (SEM) nM				Ki in Tris + 100 mM NaCl (SEM) nM			GTPgS (SEM)						Relative efficacy at μ	Ke δ (SEM) nM
		μ	δ	□	μ:δ Ratio	μ	δ	μ:δ Ratio	μ % Stim	μ EC_{50} (nM)	δ % Stim	δ EC_{50} (nM)	□ % Stim	□ EC_{50} (nM)		
Morphine		1.3 -0.3	103 -4		0.0965	149 -66	433 -43	1:03	57 -2	152 -36	28 -2	1200 -600			0.56	n/a
AAH8		0.04 (0.01) [a]	0.2 (0.02) [a]	50 (18) [a]	1:05	1.7 -0.7	1.1 -0.4	1:01	87 (3) [a]	0.9 (0.2) [a]	dns[a]	n/a[a]	dns[a]	n/a[a]	1.26	1.8 -0.1
AMB47		0.19 (0.08) [b]	0.9 (0.2) [b]	0.8 (0.1) [b]	1:05	0.4 -0.1	3.5 -0.4	1:09	96 (4) [b]	6 (3) [b]	dns[b]	n/a[b]	40 (8) [b]	>1000[b]	0.51	4.4 -0.4
AMB46		0.15 (0.08)[b]	15 (5) [b]	2 (1) [b]	0.1111	1.6 -0.2	83 -11	1:50	96 (4) [b]	2.6 (1.5) [b]	dns[b]	n/a[b]	15 (2) [b]	15 (9) [b]	0.78	95 -17

Table 2

Cmpd	ED50(SEM) mg/kg ip		
	Saline Pretreat (50C)	1 mg/kg NTX Pretreat (50C)	Saline Pretreat (55C)
AAH8	4.4(0.4)	13.7(1.6)	21.0 (0.8)
AMB47	5.3(0.3)	14.7(0.6)	64.5 (1.5)
AMB46	4.7(0.2)	12.9(1.5)	55.0 (1.3)
Morphine	4.7(0.05)	15.2(0.1)	16.4 (0.9)