ENDOMORPHIN ANALOG ANALGESICS WITH LOW ABUSE LIABILITY:
NOVEL THERAPEUTICS FOR PAIN AND OPIOID ABUSE

AN ABSTRACT

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BY

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ABSTRACT

Opioids are the most effective treatment for pain, but a host of side effects such as lethal overdoses limits their use. Endomorphins are endogenous opioid ligands that show promise as a basis for safer analgesics. Several EM analogs were developed to provide equal analgesic effects compared to morphine, with reduced respiratory depression, motor, cognitive, tolerance, and reward side effects. Tested here in reward models, morphine was compulsively self-administered and produced conditioned place preference (CPP) and locomotor sensitization after repeated injections. In sharp contrast, EM analogs were inactive in all of these models.

Mechanisms for reduced tolerance and reward are proposed here. Chronic EM analog infusions produced substantially less tolerance than equi-effective doses of morphine. Morphine upregulated glial cell markers of proinflammatory activation and signaling as well as the neuronal proinflammatory peptide CGRP. By contrast, EM analogs did not produce glial or CGRP activation suggesting reduced proinflammatory side effects. In the CPP reward model, morphine produced a place preference and decreased the cell soma size of dopamine (DA) neurons in the ventral tegmental area (VTA), a critical area for reward. EM analog 4 did not produce CPP and did not change the size of these neurons in the VTA. Penetration of the blood-brain barrier (BBB) by EM analogs was confirmed by central antagonism of the antinociceptive effects of peripherally administered analogs. This work suggests that EM analogs do not promote reward behaviors and do not produce morphological changes to DA neurons in the VTA, despite BBB penetration. Therefore, the reduced tolerance and reward side effects of
the analogs could be due to lack of proinflammatory effects and reduced DA neuron alterations.

Finally, the subjective effects of EM analogs were tested in a drug discrimination (DD) model. During DD test sessions, rats responded on the morphine-paired lever for food when pre-injected with EM analogs, indicating that the analogs were perceived as being more similar to morphine than vehicle, despite evidence that they did not produce rewarding effects. Data shown here suggest a dual role for EM analogs in the treatment of pain and opioid addiction.
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ABBREVIATIONS

µ -OR- Mu-opioid receptor
5-HT- Serotonin
ANOVA- Analysis of variance
ATP- Adenosine triphosphate
AUC- Area under the curve
BBB- Blood-brain barrier
BDNF- Brain derived neurotrophic factor
CaMKII- Ca\(^{2+}\)/calmodulin-dependent protein kinase
CGRP- Calcitonin gene-related peptide
CPP- Conditioned place preference
CREB- cAMP response element-binding protein
CRLR- Calcitonin-receptor-like receptor
DA- Dopamine
DAMGO- [D-Ala\(^2\),\(N\)-MePhe\(^4\),Glyol\(^5\)]-enkephalin
DD- Drug discrimination
DRG- Dorsal root ganglia
EM- Endomorphin
EM1- Tyr-Pro-Trp-Phe-NH\(_2\)
EM2- Tyr-Pro-Phe-Phe-NH\(_2\)
ERK- Extracellular signal-regulated kinase
FR- Fixed ratio lever pressing requirement for SA (e.g., 5 presses / infusion= FR5)
GABA- Gamma-aminobutyric acid
GFAP- Glial fibrillary acidic protein marker for astrocytes
GRK- G-protein coupled receptor kinase
HP- Hot plate antinociception test
i.t.- Intrathecal
i.v.- Intravenous
Iba1- Ionized calcium-binding adaptor molecule-1 marker for microglia
i.c.v.- Intracerebroventricular
IHC- Immunohistochemistry
IL- Interleukin
KCC2- K-Cl co-transporter-2
KO- Knockout
κ-OR- Kappa-opioid receptor
LS- Locomotor sensitization
NAc- Nucleus accumbens
NE- Norepinephrine
NFκB- Nuclear factor κB
Nlx-M- Naloxone-methiodide
NMDA(R)- N-Methyl-D-aspartate (receptor)
NOR- Nociceptin receptor (aka, orphanin receptor)
P2X- Purinergic receptors- e.g., P2X4, P2X7
PAG- Periaqueductal gray region
PBP- Parabrachial pigmented area subregion of the VTA
PBS- Phosphate buffered saline
PN- Paranigral area subregion of the VTA
pp38- Phosphorylated-p38
RAMP1- Receptor activity-modifying protein-1 for CGRP
RCP- Receptor coupling protein for CGRP
RVM- Rostral ventral medulla
SA- Self-administration
TF- Tail flick antinociception test
TLR- Toll-like receptors
VTA- Ventral tegmental area
β-Arr- Beta-arrestin
βFNA - Beta-Funaltrexamine
δ-OR- Delta-opioid receptor
Chapter 1: Background

Opiates such as morphine are a troubling problem for physicians, since they are very effective pain relievers but can lead to dependence, abuse, and lethal overdose. Over the past 10-15 years opioid overdose deaths have increased 4-fold (NIDA, 2011) along with similarly escalating addiction rates. Separation of these undesirable side effects from the beneficial analgesic effects is a major unmet goal of opioid research (Corbett et al., 2006). Several approaches are commonly taken to develop safer opioids: 1. Modifying the alkaloid structure of morphine to create novel synthetic opioids (e.g., the addition of 2 acetyl groups to the morphine structure created diacetylmorphine, also known as heroin); 2. Designing compounds that do not penetrate the BBB to avoid higher brain regions associated with tolerance, dependence, and abuse (e.g., loperamide); and 3. Developing “abuse-resistant” formulations by combining an opioid antagonist with an opioid agonist to prevent addicts from crushing and injecting the opioid or using a slow release to avoid the euphoric effects of rapid onset. These efforts have largely failed to adequately treat chronic pain conditions or they produce adverse side effects such as abuse liability. A new approach was taken here by investigating peptide-based endogenous opioids with profound analgesic effects and reduced side effects.

I. Endomorphins

Endomorphins (EM; EM1, Tyr-Pro-Trp-Phe-NH2 and EM2, Tyr-Pro-Phe-Phe-Phe-NH2) are promising endogenous candidates for the development of an opioid with
reduced side effects (Zadina et al, 1997). EM1 and EM2 are expressed in brain and spinal tissue and are thought to be involved in natural pain and reward mechanisms due to potent and selective mu-opioid receptor (µ-OR) agonist effects. Because these parent peptides are rapidly degraded, therapeutics based on these structures required development of metabolically stable EM analogs. These analogs were synthesized with the goal of providing equal or greater duration of antinociception compared to morphine with reduced side effects including tolerance, respiratory depression, cognitive/motor impairment, and abuse liability. Prescription opioid abuse is widespread among men and women (Isacson and Bingefors, 2002) and the epidemiological data shows no indication that this problem is diminishing. Therefore, a detailed abuse liability profile of the EM analogs is warranted. Mechanisms for the differential reward effects are considered here along with the potential for the analogs as therapeutics for opioid addiction.

II. Abuse Liability Models

Since no single rodent model of abuse liability can adequately mimic human addiction, EM analogs were screened in a battery of assays to develop a more thorough understanding of their potential for abuse. These tests evaluate several different facets of drug addiction including contextual associations, effects on spontaneous locomotion, direct drug consumption, workload expenditure for drug infusions, and morphine-substitution effects.

The conditioned place preference (CPP) test provides an index of contextually associated reward. During conditioning a visually distinct compartment is repeatedly paired with drug administration and another compartment with vehicle, over several days. On test day when both compartments are available to the drug-free rat, time spent
exploring the drug-paired compartment is interpreted as an indicator of reward (Tzschentke, 2007). One advantage of CPP is that rats are tested in a drug-free state, thus behavior is not influenced by the acute effects of the test compound. Reward from morphine is known to be dependent on opioid receptors since co-administration with selective opioid antagonists blocks CPP (Tzschentke, 2007). However, opioid receptors are not the only modulators of morphine CPP since glial cell inhibitors have been shown to prevent CPP to morphine and oxycodone (Hutchinson et al., 2012), suggesting inflammatory processes may contribute to reward. A final advantage of the CPP model is that locomotor behavior is measured during conditioning, providing an additional measure closely associated with reward.

One common feature among drugs of abuse is their ability to promote increases in locomotion after repeated exposure. Drugs of abuse increase locomotion through activation of the mesolimbic dopamine pathway which overlaps closely with motor pathways of the dorsal striatum. While opioids are classified as depressants, they acutely increase locomotion at low doses, produce sedation at high doses, and progressively increase locomotor behavior after chronic injections. The latter effect is known as locomotor sensitization (LS). LS (Kalivas and Volkow, 2005) can be thought of as the development of tolerance to the sedative effects of an opioid, or sensitization of brain regions that control locomotion. Both of these concepts may contribute to LS depending on the dose or route of administration. Opioid-induced LS is blocked by non-selective dopamine antagonists, selective mu and delta opioid antagonists (Vanderschuren and Kalivas, 2000), NMDA receptor antagonists (Mendez and Trujillo, 2008) and glial cell modulators (Flores et al., 2010). LS may occur through a variety of mechanisms and is a
feature of several abused opioids including morphine, oxycodone, and buprenorphine. Removal of this side effect could substantially decrease reward. Some EM analogs do not impair coordinated motor behavior on the rotarod even at equi-antinociceptive doses as morphine (Zadina et al., in preparation), however effects on spontaneous locomotor behavior and LS have not been investigated, so this was determined during place conditioning studies.

By contrast to the CPP/LS models, the self-administration (SA) model (Doherty and Frantz, 2012) more directly tests abuse liability by allowing rats intravenous access to opioid injections via lever pressings in an operant chamber. This model is more comparable to human drug consumption and most drugs of abuse, including morphine, are readily self-administered by animals. Rat SA studies consistently predict human reports of “wanting” or “liking” the drug, strongly supporting the use of SA studies to assess abuse liability (O’Connor et al., 2011). SA studies vary depending on the drug used to acquire opioid SA (e.g., cocaine is often used as a training drug), strain of rats, or length of access of the SA sessions. Long-term access SA (e.g. 12 hour/day) models (Doherty et al., 2009) are often used to mimic real-world drug taking and are the most sensitive model for rats to acquire opioid SA. In long-term models, opioids such as heroin, fentanyl, and oxycodone are self-administered to a greater extent than during short-term access (Beardsley et al., 2004; Wade et al., 2015). This suggests that long access increases the rate of opioid SA. Therefore, a long-term access SA model was used to compare EM analogs and morphine.

The drug discrimination (DD) model (Krivsky et al., 2006) has an advantage of comparing a known drug of abuse, such as morphine, with experimental compounds that
may share common pharmacological targets. In this assay, rats are repeatedly trained to discriminate a training dose of an abused drug (e.g., morphine 3.2 mg/kg, s.c.) from vehicle injections for food pellet rewards in an operant chamber. For example, morphine is paired with the left lever and vehicle with the right lever in half the rats, and other rats are counterbalanced vice versa. After a series of training criteria are met, test sessions are administered in which both levers deliver food pellets. This requires the rats to use the cue associated with the training compound to prompt lever responding. Test compounds are administered across a range of doses and rats make associations with these injections by pressing either the drug- or vehicle-associated lever. Other advantages of DD include measuring food-reinforcement rates (low rates indicate behavioral disruption), time-course analyses, and agonist-antagonist interactions. A drug which lacks abuse liability in classic CPP, LS, and SA models, but substitutes for a known drug of abuse (such as morphine) in the DD model, is a promising candidate for the treatment of opioid addiction. Therefore, the substitution effects of EM analogs was studied in rats trained to discriminate morphine.

Each of these tests provide a unique measure of drug abuse potential. The CPP, SA, and DD models evaluate the environmental association of a drug, drug consumption rates, and drug substitution potential, respectively. SA and LS models have been argued to most accurately predict human drug abuse liability (O'Connor et al, 2011; Robinson and Berridge, 2003; Tzschentke et al, 2002). CPP provides a measure of drug-seeking behavior in a non-drug induced state, while DD investigates drug-substitution effects in morphine-experienced rats. Data generated from these models aim to provide a thorough
evaluation of the abuse liability of EM analogs and their application for the treatment of pain and opioid addiction.

III. Blood-Brain Barrier (BBB) Penetration Considerations

The BBB penetration of EM analogs is unknown, and it is unclear whether lack of access to centrally located reward regions after peripheral injection contributes to the low side effect profile of EM analogs. While peripheral opioid receptors contribute to the antinociceptive effects of various opioids, it is likely that some degree of BBB penetration is necessary to induce thermal antinociception. Naloxone methiodide (Nlx-M) is an opioid receptor antagonist that cannot penetrate the BBB. Central injection (intracerebroventricular, i.c.v.) of Nlx-M blocks the antinociceptive effects of peripherally administered morphine (Al-Khrasani et al., 2012). Thus morphine likely crosses the BBB to produce antinociception. While peripheral restriction is one strategy to reduce the rewarding effects of an opioid, peripherally restricted agents such as loperamide do not make effective analgesics for intractable pain conditions.

BBB penetration can also be assessed by using the hot plate (HP) test since this test measures centrally mediated responses such as licking or shaking the paw. Opioids that penetrate the BBB reliably inhibit HP latencies in this model, suggesting they modulate the descending pain pathway via central regions such as the PAG. Since central opioid receptors are required to block HP responses, this test was used to study central mechanisms underlying central antinociception from EM analogs. Therefore, to determine if the antinociceptive effects of EM analogs require central µ-ORs, the selective irreversible µ-OR antagonist β-Funaltexamine (βFNA) was used.
Finally, the DD test, described above, is a third behavioral indicator of BBB penetration. Rats are required to use centrally located mechanisms in order to discriminate injections of morphine from vehicle since the operant training requires use of the drug cue to prompt correct lever responding. A test drug which substitutes for morphine (e.g. rats press the morphine-paired lever for food) during DD test sessions would likely penetrate the BBB since the test drug would require CNS access to prompt morphine-lever responding. For example, loperamide does not penetrate the BBB and does not substitute for morphine in the DD model (Gianutsos and Harbans, 1975). Likewise, opioids such as fentanyl readily substitute for morphine since fentanyl crosses the BBB to produce centrally mediated opioid effects (Craft et al, 1999).

**IV. Opioid Receptors and Signaling**

Opioid receptors are found throughout the brain and spinal cord and broken down into 4 main classes. The mu opioid receptor (μ-OR) is the major target for suppressing pain and as described above, is the target for EMs. Delta (δ-OR) and kappa (κ-OR) opioid receptor agonists can also produce antinociception which can be blocked by selective antagonists, however δ-OR agonists can produce convulsions and κ-OR agonists have well-known dysphoric effects that limit these receptors as clinical drug targets. Finally, the nociceptin receptor (NOR) regulates pain and emotional behaviors upon activation by the endogenous NOR ligand nociceptin/orphanin FQ. This peptide has been shown to be involved in anxiety, depression, and addiction behaviors. The various opioid receptors have clear and diverse roles in pain and analgesia, however μ-OR agonism is critical for inducing prolonged analgesia. μ-ORs are heavily expressed in spinal cord, raphe nuclei, ventral tegmental area, PAG, and higher brain regions such as the amygdala and
hippocampus. Thus, µ-ORs are expressed in critical regions associated with the brain’s “pain matrix” (Legrain et al., 2011). Furthermore, µ-ORs co-localize in many of these regions with both EM1 and EM2, suggesting these peptides are properly located to be endogenous ligands for µ-ORs (Greenwell et al., 2007; Martin-Schild et al., 1999).

The main biophysical features of the µ-OR include a 7 transmembrane receptor coupled to α, and βγ G-protein subunits. µ-ORs are G_{i/o} coupled so activation by ligands generally produces inhibitory cellular responses. When opioids bind to the µ-OR the α subunit dissociates from the β and γ subunits and stimulates G-protein coupled receptor kinases (GRKs). Christie has suggested that phosphorylation of µ-ORs by GRKs stimulates interaction with beta-arrestin (β-Arr) proteins that are critical for receptor desensitization and internalization after chronic opioid exposure (Christie, 2008). β-Arr dissociates the receptor from G-protein signaling and is a key signaling protein that modulates tolerance to morphine. In one example, genetic knockout (KO) of β-Arr attenuated tolerance produced by chronic morphine and β-Arr -KO mice showed increased antinociceptive effects from morphine (Bohn et al., 2000). β-Arr appears critical for the analgesic tolerance produced by morphine and has indeed been investigated for novel drug development (DeWire et al., 2013). However, this signaling pathway appears complex since β-Arr –KO mice also show increased sensitivity to the rewarding effects of morphine, including increased striatal dopamine release compared to control mice (Bohn et al., 2003).

Several opioids that diverge in β-Arr recruitment and receptor internalization effects may explain their distinct effects in vivo. For example, low efficacy morphine produces very little internalization of µ-ORs, in contrast to high efficacy [D-Ala^2,N-
MePhe⁴,Glyol⁵]-enkephalin (DAMGO) which rapidly internalizes μ-ORs (Christie, 2008). Morphine does not internalize μ-ORs efficiently but desensitizes receptors rapidly, whereas DAMGO quickly internalizes and more efficiently re-sensitizes the receptor for signaling. Since DAMGO and other high efficacy agonists produce significantly less tolerance than morphine it is possible that preferential μ-OR internalization and recycling by DAMGO may explain the favorable tolerance profile since there would likely be more functional opioid receptors available after chronic exposure. The differential in vivo profiles of DAMGO and morphine is consistent with the receptor trafficking studies.

The idea that two agonists can induce differential receptor conformations or phosphorylation even though the agonists share the same receptor target is known as functional selectivity (Kenakin, 2007). Simply put, the agonist can be thought of as a “modulator” of a receptor which has many functions depending on the agonist bound and concentration of agonist. The receptor is analogous to a “conduit” for transmission of energy, whereas signaling proteins such as GRKs can be thought of as a revolving cast of “guests” that vary depending on the agonist (Kenakin, 2007, 2010). A recent hypothesis proposes that biased agonists induce unique conformations of the receptor that may recruit different subsets of GRKs resulting in differential phosphorylation patterns similar to “barcodes” (Wisler et al, 2014). These “barcodes” may induce different β-Arr conformations that activate distinct cellular functions. Overall, functional selectivity may partially explain differences between a variety of opioid agonists including the EMs (Rivero et al, 2012) and their ability (or inability) to produce adverse side effects such as tolerance, dependence, and reward. There are numerous in vitro screening models designed to detect biased agonism, but how these models translate to in vivo systems is
unclear. The unique structures of the analogs may provide interesting tools for testing these concepts due to their diverse pharmacodynamic effects and high µ-OR selectivity.

V. Tolerance to chronic opioid infusions: high vs. low potency agonists

Tolerance is a persistent problem that complicates pain therapy due to loss of analgesic effectiveness by chronic opioids. Tolerance to morphine occurs rapidly, requires patients to take increasingly larger doses to manage pain, and contributes to reward effects. Intrathecal (i.t.) delivery models infuse opioids via a mini-pump connected to a catheter implanted in the subarachnoid space of the lumbar spinal cord. Antinociceptive tests show that tolerance to i.t. mini-pump infusion of morphine occurs in as few as 2-3 days on standard hot plate or tail flick tests. High efficacy mu-agonists such as DAMGO or sufentanyl produced less tolerance than low efficacy morphine in this model (Stevens and Yaksh, 1989b). Due to high efficacy µ-OR agonist effects the EM analogs may produce less tolerance than morphine. While efficacy is one consideration for an opioid with reduced tolerance, reduced immune reactivity through neuron-neuropeptide-glial activation may hold the key to understanding the differential effects of chronic morphine and EM analogs.

VI. CGRP induced glial cell activation promotes tolerance to morphine-induced analgesia

The neuropeptide calcitonin gene-related peptide (CGRP) plays a critical role in a variety of physiological functions including autonomic, learning, motor activity, vasodilation, and nociceptive functions. CGRP is broadly expressed in central nervous
system regions including cerebral cortex, hippocampus, amygdala, periaqueductal gray, dorsal raphe nuclei, brainstem motor nuclei, and the spinal cord dorsal horn (Ma et al, 2003). Peripheral nervous system regions such as the dorsal root ganglia (DRG) contain dense CGRP fibers that project to the dorsal horn where CGRP receptors are localized on a variety of cell types including microglia, astrocytes, and neurons. G-protein coupled receptors for CGRP are unique since they contain 3 proteins: the seven-transmembrane calcitonin-receptor-like receptor (CRLR), a chaperone protein called receptor activity-modifying protein-1 (RAMP1), and a receptor coupling protein (RCP) that is necessary for signal transduction (Ma et al, 2003). RCP, RAMP1, and CRLR co-label strongly with CGRP in spinal and peripheral ganglia and it has been proposed that CGRP receptor activation promotes spinally mediated inflammation, pain, and tolerance to opioids (Wang et al, 2010). Indeed, efforts are underway to develop CGRP antagonists as pain relievers and to attenuate tolerance to morphine (Hirsch et al, 2013). For example BIBN4096BS, a CGRP antagonist, has been shown to inhibit tolerance to morphine by reducing crosstalk between CGRP neurons and glial cells. Therefore, CGRP interacts with neuronal and immune cells to promote pain and tolerance to opioids.

A trisynaptic hypothesis of how CGRP facilitates tolerance to chronic morphine has been proposed to involve neurons, microglia, and astrocytes (Wang et al, 2009). Spinal CGRP interacts with its receptor components located on microglia that upregulate phosphorylation of the key microglia signaling kinase p38. This kinase initiates translocation of nuclear factor κB (NFκB) into the nucleus to increase mRNA for cytokines such as IL-6. IL-6 promotes pain and tolerance to opioids together with IL-1β released by local astrocytes through an ERK-phospho-Stat pathway. These cytokines
promote inflammation, pain, and hyperalgesia from chronic opioid exposure. Together with CGRP, IL-1β and IL-6 act at their respective receptors to increase Ca^{2+} production. This leads to the increased CaMKII and CREB production necessary for NMDA receptor transcriptional components known to counteract the antinocicptive effects of opioids. Thus, CGRP activation of NMDA receptors is a contributor to morphine tolerance. For example, co-administration of NMDA receptor antagonists reduced tolerance to morphine (Wang et al., 2010). Since morphine readily promotes CGRP release, glial reactivity, and the rapid development of tolerance, EM analogs were tested in these models and compared to morphine after undergoing the i.t. tolerance model described above.

**VII. Purinergic receptors (P2X) modulate glial cell activation, hyperalgesia, and tolerance after chronic morphine**

An emerging line of research suggests involvement of several ATP-stimulated purinergic receptor subtypes in the development of paradoxical pain and tolerance to chronic opioids. There are 5 major purinergic receptors subclasses: P2Y, P2Z, P2U, P2T, and P2X. The P2X subclass is sparingly expressed on resting microglia or astrocytes, but injury or chronic morphine exposure strongly upregulates ATP-stimulated P2X4 and P2X7 receptors in microglial cells. Horvath and colleagues showed P2X4 receptors co-labeled with a marker for activated microglia (OX-42) in rats given chronic morphine (Horvath et al., 2010b). They proposed that morphine binds to µ-ORs located on spinal microglia, inducing activation of P2X4 that stimulates cytokine and chemokine release through the phosphorylated-p38 pathway described above. The model hypothesizes that chronic morphine increases neuron excitability and promotes hyperalgesia, or
“paradoxical pain”, by decreasing neuronal KCC2 activity, increasing intracellular Cl̅, and increasing microglial-derived BDNF signaling in the spinal dorsal horn (Ferrini et al., 2013).

While P2X4 receptor modulation by morphine has been studied most extensively (Horvath et al., 2010b), other P2X subtypes may contribute to tolerance produced by morphine. For example, morphine-tolerance was blocked by a selective P2X7 antagonist Brilliant Blue G [BBG] (Zhou et al., 2010). Pre-treatment with BBG prevented morphine-induced tolerance and microglial activation, and interference with P2X7 mRNA attenuated tolerance from morphine (Zhou et al., 2010). Since ATP is the endogenous ligand for P2X7 receptors it is possible that chronic morphine increases the ATP-stimulated inflammation. Chronic morphine also increases excitatory glutamate signaling at NMDARs which can create a positive feedback loop of glutamate and ATP-P2X7 stimulation that exacerbates tolerance. Activation of P2X7 receptors induces IL-18 and TNFα cytokine release through downstream microglial-p38 signaling mechanisms described above (Chen et al., 2012). IL-18 and TNF have been linked to both neuropathic and inflammatory pain conditions. While production of IL-18 occurs in microglial cells, astrocytic cells contain the IL-18 receptor, so IL-18 is an important cytokine that induces glial activation. Indeed, D-serine, a modulator of NMDARs, is upregulated by chronic morphine through IL-18 activation (Chen et al., 2012). Blockade of D-serine reduced tolerance to morphine so it is possible that D-serine released by astrocytes engages astrocyte-neuron communication via IL-18 to stimulate mechanisms that increase NMDAR activation. Since ATP-P2X7 receptor activation is linked to morphine-tolerance, and the p38 pathway has been shown to be a common target of these
proinflammatory pathways, EM analogs were tested and compared with morphine in these models.

**VIII. Activation of glial cell toll-like receptors counteracts the antinociceptive effects of morphine**

There has been a recent surge in reports describing glial cell modulation of the antinociceptive and rewarding effects of opioids. Evidence that morphine and other opioids bind receptors located on astrocytes and microglia suggest these opioids are recognized as foreign substances to immune system cells. Hutchinson and colleagues (2011) described distinct binding pockets for morphine located on toll-like receptors (TLRs) and suggested a non-neuronal-, and non-µ-OR- mechanism for tolerance, respiratory depression, withdrawal, and reward (Hutchinson *et al*, 2011). Specifically, *in silico* binding studies show that morphine binds the TLR4-MD2 complex leading to phosphorylation of p-38 (pp-38) to engage proinflammatory responses. For example, morphine is more potent in TLR4 knock out mice compared to wild types (Hutchinson *et al*, 2010b). Blockade of TLR4 receptors with (+)-naloxone, a compound devoid of opioid activity but capable of antagonizing TLR receptors, attenuated tolerance to morphine (Eidson and Murphy, 2013a, b) and increased the potency of acute morphine analgesia (Hutchinson *et al*, 2010b). Chronic morphine regimens upregulated astrocytic (GFAP) and microglial signaling markers (pp-38 and OX-42) in the spinal cord (Horvath *et al*, 2010a), medulla, periaqueductal grey (Eidson *et al*, 2013b; Hutchinson *et al*, 2009; Tawfik *et al*, 2005) and in the VTA (Hutchinson *et al*, 2009). These studies suggest that glial cells in these regions counteract the antinociceptive effects of chronic morphine to produce tolerance and reward (see below). Therefore, glial cell activation markers were
studied in rats given chronic infusions of EM analogs and compared with those given morphine.

**IX. Mesolimbic reward mechanisms of opioids**

The mesolimbic reward circuit is comprised of dopaminergic neurons originating in the ventral tegmental area (VTA) that project to ventral striatal, neo-cortical, and hippocampal areas in the forebrain. Rewarding effects of morphine and other opioids occur via disinhibition of VTA-dopamine (DA) neurons that project axons to the nucleus accumbens (NAc). Opioids bind to receptors on GABA neurons in the VTA which normally exert inhibitory control over nearby DA neurons. Binding of opioids to μ-ORs located on GABA neurons disinhibits DA release, thereby increasing DA transmission and promoting reward. Long term use of morphine or heroin is associated with impaired DA function and tolerance to the rewarding effects. Human and animal studies consistently show chronic use of morphine or heroin (diacetylmorphine) impairs DA transmission and produces visible reductions in the size of DA cells in the VTA (Kish *et al*, 2001; Mazei-Robison *et al*, 2011). In rats, chronic opioids shrink DA cell soma sizes in the VTA for at least 2 weeks after morphine withdrawal (Chu *et al*, 2007), and this has been closely associated with tolerance to the rewarding effects of opioids (Berhow *et al*, 1995; Chu *et al*, 2008; Mazei-Robison *et al*, 2011; Sklair-Tavron, 1996; Spiga *et al*, 2005). Hyperexcitability of DA neurons and decreased DA output occur concurrently with DA cell soma shrinkage in the VTA (Russo *et al*, 2007). This may contribute to compulsive opioid seeking displayed by dependent users as a compensatory mechanism for reduced DA release. An opioid that lacks reward effects may not produce these morphological and neurochemical changes to DA neurons after chronic administration.
Since DA soma shrinkage in the VTA is a hallmark of chronic morphine usage in rodents and humans, it was hypothesized that EM analogs would not reduce the size of DA neurons, consistent with their low abuse potential.

**X. Rewarding effects of opioids are modulated by glial cells**

While the spinal effects of chronic opioids on glial cells is well-studied, the influence of glial cells is not well characterized in supra-spinal regions. Some reports demonstrate glial cells modulate the rewarding effects of opioids through similar mechanisms described for spinal tolerance. Behavioral studies demonstrate that astrocyte and microglial inhibitors block the rewarding and respiratory depressive effects of morphine (Hutchinson et al., 2008; Narita et al., 2006). Furthermore, supra-spinal glia appear to modulate the rewarding effects of morphine since microglia cells labeled with ionized calcium binding adaptor molecule 1 (Iba1) were upregulated in the nucleus accumbens (NAc) shell after chronic morphine injections, and blockade of NAc microglia reversed the rewarding effects of morphine (Zhang et al., 2012). In the VTA, the rewarding effects of morphine were shown to be influenced by fibroblast growth factor-1 (FGF-1) receptors (FGFR-1) located on astrocytes (Flores et al., 2010). This model suggests morphine upregulates NMDAR-1 levels in the VTA due to FGF-1 signaling between astrocytes and neurons. Blocking FGF-1 signaling reduced rewarding locomotor sensitization behaviors by morphine through this mechanism. Together these studies provide *in vivo* evidence that the rewarding effects of morphine are influenced by non-opioid binding sites located on glial cells in supra-spinal regions. Future testing will be necessary to determine whether EM analogs induce glial cell signaling in supra-spinal regions such as the VTA.
**Brief Summary**

The main goals of this dissertation were to test the abuse potential of EM analogs in an extensive battery of classic behavioral models of addiction, determine if the analogs penetrate the BBB, and propose mechanisms for the differential effects of the EM analogs compared to morphine. The overall hypothesis was that, since the structures of the analogs are based on naturally occurring EM peptides which have high µ-OR selectivity, chronic administration would produce a different profile of effects from those induced by morphine and related opium-derived compounds. Two potential mechanisms for the distinct profiles are 1) differential glial cell-related inflammation known to contribute to tolerance and reward, and 2) changes in dopaminergic neurons in the VTA, known to affect reward-tolerance. Consistent with the overall hypothesis, unlike morphine, the analogs did not induce reward behavior, glial activation or alter VTA DA neurons despite evidence that the analogs penetrate the BBB. Finally, the potential use of EM analogs for opioid maintenance therapy was discussed. An ideal candidate for the treatment of opioid abuse would lack abuse potential in multiple models, produce less tolerance, glial cell activation, respiratory suppression, and not impair or sensitize locomotion. This work demonstrated that EM analogs appear to meet these criteria as novel opioids with reduced side effects.
Chapter 2: Lack of conditioned place preference, locomotor sensitization, and dopamine cell alterations by EM analog 4 suggests low abuse liability

INTRODUCTION

The motivation to take addictive drugs such as opioids is a central question for researchers attempting to develop drugs that do not engage reward circuits. Drugs of abuse produce powerful contextual associations with environmental surroundings and reinforcing drugs produce Pavlovian conditioned behaviors after repeated drug-context pairings. The conditioned place preference (CPP) test reliably detects reward behavior induced by opioids (Tzschentke, 2007) and other drugs of abuse in rodents. By pairing drug infusions with visually distinct stimuli over several conditioning sessions, animals spend more time in contexts previously paired with injections of morphine. CPP tests animals in a non-drug induced state, so the behavior is not directly influenced by acute drug effects. By contrast to CPP, locomotor sensitization (LS) effects are tested while animals are under the influence of the drug. LS is a classic behavioral effect of opioids defined by progressively increasing spontaneous motor behavior after repeated drug exposures. Preliminary studies have shown that EM analogs do not produce CPP effects after 3 pairings (Zadina et al, in preparation) or after intracerebroventricular administration. This experiment extends these findings to 5 daily pairings of vehicle, EM analog 4, or morphine in LS and CPP models.
Following the LS and CPP experiments, dopamine (DA) neuron cell somas of the VTA were analyzed in these rats, since chronic morphine has been shown to visibly shrink the size of these neurons in mice, rats, and in postmortem human opioid-dependent users (Chu et al., 2008; Mazei-Robison et al., 2011; Russo et al., 2007; Sklair-Tavron, 1996; Spiga et al., 2003). These studies show that in a variety of species, chronic morphine reduced area and volume of DA neurons stained with tyrosine hydroxylase (TH), the rate limiting enzyme necessary for DA, in the ventral tegmental area (VTA). The posterior VTA (pVTA) region was chosen for analysis because dense DA cells are present here. The parabrachial pigmented area (PBP) and the paranigral area (PN), subregions of the pVTA, project axons to the nucleus accumbens and other forebrain regions associated with reward (Ikemoto, 2007), so these subregions were used for analysis. In addition to long lasting morphological alterations, these neurons show hyperexcitable firing rates and blunted DA release induced by chronic morphine. These electrophysiological, neurochemical, and morphological changes have been closely associated with reward and dependence produced by morphine. The following comparative analysis of DA cell soma sizes was performed in pVTA tissue from rats injected with vehicle, morphine, or EM analog 4 during CPP conditioning. DA neuron morphology reductions were expected to coincide with a rewarding drug such as morphine, but not EM analog 4.

METHODS

Subjects: Male Sprague Dawley rats were purchased from Charles River. Rats were tested at approximately 275-350g (surgery at 65d old, test at 70-80 days old). Rats were habituated to the colony room for at least 5 days prior to behavioral testing and room
temperature was maintained at 22°C. All rats were housed 2-3 per cage during habituation and single housed after surgery, under a 12:12 light/dark cycle (lights on at 07:00). Experiments were conducted during the light cycle. All procedures were approved by the Tulane Institutional Animal Care and Use Committee (IACUC) and conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Intravenous catheter implantation: Rats were catheterized in the left jugular vein in a similar manner as others (Doherty et al, 2012) with minor modifications. Rats were anesthetized with an isoflurane/oxygen mixture (4-5% induction, and 1.5-2.5% for the remainder of the surgery). A 1 cm area on the ventral and a 2-3 cm area on the dorsal side of the rat were shaved and cleaned for incision. The catheter was passed subcutaneously from the back, inserted into the left jugular vein, and secured with sutures. Wounds were sutured and dressed with antibiotic ointment and rats were given a subcutaneous injection of 0.5% lidocaine and 0.25% bupivacaine to treat topical pain. All rats were allowed 5 days to recover from surgery prior to behavioral testing. Catheters were flushed daily with 0.1 ml of streptokinase (0.067 mg/ml) to maintain catheter patency. Rats with questionable catheter patency were tested with an injection of the ultra-short acting barbiturate anesthetic, methohexital (0.1 ml of 10 mg/ml). If muscle tone was not lost within 3 seconds, the catheter was considered faulty and the rat was excluded from the analysis.
Chemicals: Peptides were synthesized by standard solid phase methods at 1 mMol on Rink amide resin via Fmoc chemistry with purity and sequence identity confirmed by HPLC and MS. Three analogs selected for full characterization were synthesized at 2g scale by American Peptide Company (Sunnyvale, CA). Morphine sulfate was supplied by NIDA.

Conditioned Place Preference (CPP): Baseline activity was measured in standard CPP chambers (TSE; Chesterfield, MO) over 2 days with 4 trials per day (2 in the morning and 2 in the afternoon) lasting 20 mins each. Conditioning trials were conducted immediately after injection of drug (morphine or analogs) or vehicle (20% PEG in saline) and rats were confined to distinct compartments (striped vs. gray walls) for 30 mins. Doses of morphine and analogs (1.8, 3.2, and 5.6 mg/kg, i.v.) were chosen based on tail flick antinociception producing MPE levels of 70-80%, 100%, and a ¼ log dose higher to test submaximal, maximal, and supramaximal doses, respectively. Conditioning trials were conducted for 5 days and tested in an unbiased fashion such that drug/environment pairings were counterbalanced for time of drug injection (am or pm) and compartment (preferred or non-preferred) baseline activity. One day following the last conditioning trial, a 20 min test trial was administered in the same manner as the baseline trials in which rats were free to explore both compartments. Change in time spent exploring the drug-paired compartment (test-baseline) indicated reward potential. Approximately 20 mins following the final test session, rats were perfused and brain and spinal cord samples were taken for immunohistochemical analysis.
Immunohistochemistry (IHC): Rats were anaesthetized with a mixture of ketamine/xylazine (85/10 mg/kg, i.p., respectively) and perfused intracardially first with 200ml of 0.1mol/L phosphate buffered saline (PBS) immediately followed by 300ml of 4% paraformaldehyde in 0.1mol/l PBS (pH= 7.4). Brains were removed and post-fixed at 4°C in the same fixative for 18h. After post-fixation, brains were incubated in 30% sucrose at 4°C for 2 days and sectioned coronally on a cryostat at 40µm at the level of the pVTA (Chu et al, 2008; Spiga et al, 2003). After 2 consecutive washes in PBS, sections were blocked with 5% normal donkey serum (NDS) for 1h, and incubated with the primary antibody anti-tyrosine-hydroxylase (anti-TH, 1:3000 Cell Signaling Technology, Danvers, MA) overnight at 4°C. Slices were washed twice in PBS, re-blocked for 1 h, and incubated with the secondary antibody Alexa594, for 2h (Life Technologies Carlsbad, CA). Sections were washed, mounted on slides with Prolong Gold (Life Technologies), and stored at 4°C. Posterior VTA sections were verified according to the atlas of Paxinos and Watson (1998). Images of the PBP and PN subregions of the pVTA were captured in z-stacks (1µm) with at least 5 tissue slices per rat and 5-6 rats per drug group. Stereo Investigator software (MBF Bioscience; Williston, VA) was used to quantify soma size using the optical fractionator probe to survey a sample of neurons in each z-stack while the nucleator probe was used to measure the cross-sectional area and volume of each cell soma. The optical fractionator probe was used to quantify the number of cells in a particular section of tissue through systematic random sampling. Between 12 and 16 regions were surveyed per z-stack. While the optical fractionator probe utilized stereological techniques to select a random sample to be analyzed, the nucleator probe measured each selected neuron’s cross-sectional area. Therefore, the simultaneous use of
these probes systematically assessed neurons in the PBP and PN in each subject, and provided morphological data for these cells including surface area ($\mu m^2$) and volume ($\mu m^3$). Neurons were eligible to be quantified if they were located within the counting frame and/or if their soma touched either of the nucleator frame’s green borders. Neurons were ineligible if located outside the counting frame and/or if their soma touched the frame’s red borders. After determining this optimum depth, the center of the neuron’s soma was located and analyzed by the nucleator probe. To eliminate bias associated with tissue orientation, nucleator rays were randomly arranged between quantifications. The nucleator probe accounted for tissue thickness and the cross-sectional area to determine surface area and volume of cell bodies. All images and data analyses were collected by a blinded investigator.

Data analysis: CPP and DA soma size data were analyzed using 1-way analysis of variance (ANOVA) with Newman-Keuls post-hoc comparisons (GraphPad Prism, San Diego, CA). Locomotor data recorded during the conditioning sessions were analyzed by 2-way ANOVA (session x drug). Locomotor data from the first session were subtracted from the final session to assess locomotor sensitization.

RESULTS

**Conditioned place behavior and locomotor effects.** Morphine produced conditioned place preference (CPP) effects after 5 days of conditioning ($F[3, 25]= 4.173, p=0.0159$) with the 3.2 mg/kg dose ($p< 0.05$, Figure 2.1). The 1.8 and 5.6 mg/kg doses of morphine did not produce CPP. EM analog 4 did not produce CPP (or aversion) at any
dose (F[3, 29]= 0.9523, p=0.4283 n.s.). Acute morphine and EM analog 4 produced similar antinociceptive effects on the tail flick (TF) test 20 minutes after injection. Hence during the same timeframe that drug conditioning occurred, rats were given equi-antinociceptive doses of morphine or EM analog 4, yet analog 4 did not produce CPP.

**Figure 2.1 Conditioned place behavior and antinociceptive effects of morphine and EM analog 4.** The left figure shows conditioned place preference (CPP) effects for morphine (3.2 mg/kg, i.v.) after 5 days of drug-environment pairings. By contrast, EM analog 4 did not show conditioned place preference or aversive effects. The right figure shows tail flick (TF) % maximal possible effect (%MPE) 20 mins after injection of morphine or analogs. Nearly identical antinociceptive effects of morphine and analog 4 were produced during the same time frame rats underwent CPP conditioning. +p< 0.05 compared to vehicle.

Daily morphine injections promoted locomotor sensitization (LS) as measured by increased distance traveled relative to controls (treatment effect: F[3,28]=7.493, p=0.0008; day effect: F[4, 112]=13.16, p< 0.0001; interaction F[12,112]=4.131, p< 0.0001, Figure 2.2). The 1.8 mg/kg dose of morphine increased locomotion across all sessions, whereas higher doses (3.2 and 5.6 mg/kg) initially suppressed locomotion, followed by a gradual increase indicative of LS. EM analog 4 did not produce LS at any dose tested (F[4, 36]= 1.6, p=0.1956, n.s.). When comparing the difference between day 5 and day 1, morphine (F[6, 47] = 7.635, p < 0.0001), but not EM analog 4, produced
significant LS. Post-hoc comparisons showed that, compared to vehicle, morphine produced LS at all doses tested (1.8 and 3.2 mg/kg, \( p < 0.001 \), 5.6 mg/kg, \( p < 0.01 \)), while EM analog 4 did not produce LS at any dose tested (\( p = n.s. \)).

**Figure 2.2 Locomotor effects of morphine and EM analog 4.** Activity was measured in meters during 5 daily sessions conducted immediately after drug injection (i.v.), or during the final non-drug test session. **a.** Locomotor effects of morphine differed by dose with 1.8 mg/kg producing both acute increases and chronic locomotor sensitization, while 3.2 - 5.6 mg/kg initially suppressed locomotion and then induced chronic sensitization. **b.** Locomotor effects of analog 4 were no different from controls. **c.** Subtracting day 1 locomotion from day 5 shows morphine produced LS, but EM analog 4 did not. **d.** Locomotor behavior measured during the non-drug test session shows morphine and analog 4 did not differ from controls. +, ++, +++ \( p < 0.05 \), < 0.01, < 0.001 compared to vehicle; *, **\( p < 0.05 \), < 0.01 compared to morphine.
**VTA DA neuron morphology analysis.** Rats injected with morphine showed a dose-dependent reduction in size of dopamine (DA) neurons in the posterior VTA (Figure 2.3). Surface area ($F[2, 27]$=6.096, $p$= 0.0065) and volume ($F[2, 27]$= 4.185, $p$= 0.0261) of TH-positive somas were reduced by morphine (5.6 mg/kg, i.v., $p$< 0.05). By contrast, EM analogs did not alter either the surface area ($F[3, 32]$= 0.9463, $p$= n.s.) or volume ($F[3,32]$= 0.9590, $p$= n.s.) of DA neurons in the pVTA. Thus, chronic infusions of EM analogs did not alter DA soma sizes in the VTA in the same model where morphine produced CPP and LS effects and reduced DA soma size in the VTA.
Figure 2.3 Chronic morphine, but not EM analog 4, reduced DA cell surface area in the posterior ventral tegmental area (pVTA). a. Low magnification section of pVTA used for analysis of DA morphology. Rats were perfused after the final CPP test session and pVTA sections were stained with tyrosine hydroxylase (TH) by IHC. TH+ somas from z-stacks were analyzed by MBF Stereo Investigator software for surface area (µm²) and volume (µm³) in the parabrachial pigmented area (PBP) and paranigral area (PN) of the pVTA. b. An example in PBP neurons where morphine (5.6 mg/kg, i.v.) reduced the surface area and volume of cell somas, while EM analog 4 did not alter soma sizes. c-f. Surface area and volume of cell somas quantified in PBP and PN regions using the Stereo Investigator nucleiator probe. Scale bars=50µm (a) or 10µm (b). 6-8 cells were quantified per rat with 5-6 rats per drug group. \(+p<0.05\) compared to vehicle.
DISCUSSION

This experiment found that conditioning with EM analog 4 did not produce conditioned place preference (CPP) or locomotor sensitization (LS) in the same model where morphine produced CPP and LS effects (Figures 2.1 and 2.2). The antinociceptive effects of i.v. EM analogs (1.8 - 5.6 mg/kg) were similar to i.v. morphine in the tail flick test 20 mins after injection, when rats were likely experiencing peak drug effect during conditioning. Morphine gradually produced locomotor sensitization (LS) during conditioning, while EM analog 4 did not. One unique finding in this experiment is that the low dose of morphine (1.8 mg/kg) increased locomotion in the first session and progressively increased locomotion in the remaining sessions. The middle and high dose of morphine (3.2 and 5.6 mg/kg) initially suppressed locomotion, then increased locomotor behavior by the final sessions. By contrast, EM analog 4 did not produce LS at any dose tested consistent with the lack of CPP. This supports the hypothesis that LS effects of morphine promote drug-seeking behavior (Robinson et al, 2003), but not EM analog 4. The current study extends these findings because DA morphological abnormalities in the posterior ventral tegmental area (pVTA) were found in rats given morphine, but not EM analog 4 (Figure 2.3). Immunohistochemical analysis showed that morphine injections produced a dose-dependent decrease of the area and volume of dopamine neurons in the pVTA. By contrast, daily injections of EM analog 4 did not alter pVTA soma sizes. Thus, morphine tested positive on 3 paradigms associated with rewarding drug properties (CPP, LS and DA soma shrinkage) while equi-antinociceptive doses of analog 4 did not. The results suggest clinical utility for analog 4 as a novel pain reliever with reduced abuse liability.
There are several potential reasons why EM analog 4 did not produce CPP. One simple explanation is that EM analog 4 did not elicit LS during conditioning, while morphine induced a robust LS effect. LS is associated with drug-seeking behavior in a variety of reward models (De Vries et al., 1998; Robinson et al., 2003; Tzschentke et al., 2002). For example, reinstatement of heroin self-administration has been associated with the expression of LS since animals that previously self-administered heroin showed exaggerated locomotor responses upon challenge with heroin, cocaine, and amphetamine (De Vries et al., 1998). Another study showed LS was not observed during tramadol conditioning, however tramadol produced CPP effects (Tzschentke et al., 2002), and clinical reports show that tramadol has reduced abuse liability in non-dependent populations, although some propensity for abuse of tramadol has been found in opioid-dependent populations (Cicero et al., 1999). Since EM analog 4 did not produce LS, CPP, or aversive effects, this experiment supports the hypothesis that analog 4 has low abuse liability relative to other opioids.

Most studies use relatively high, dependence-inducing doses of morphine to produce DA soma shrinkage (Chu et al., 2008; Chu et al., 2007; Mazei-Robison et al., 2011; Spiga et al., 2003), however the current experiment utilized physiologically comparable antinociceptive doses of morphine and EM analog 4 to determine the threshold for the production of DA soma shrinkage. It’s important to note that this effect also occurred in the VTA tissue sampled from post mortem human heroin users (Mazei-Robison et al., 2011), so the animal studies are translational to human drug use. Analysis of dopamine neurons indicated that 5 daily injections of morphine (5.6 mg/kg), but not any dose of EM analog 4, reduced the size of DA neurons in the pVTA. The pVTA
neurons synapse in the nucleus accumbens (NAc) and release DA at median spiny neurons that project to prefrontal cortex regions such as the anterior cingulate cortex (Ikemoto, 2007). Opioids likely bind to mu-opioid receptors (µ-ORs) located on inhibitory GABA-containing neurons in the pVTA, and upon binding disinhibit DA neurons to stimulate action potential firing and subsequent DA release in the NAc, an effect associated with reward. Chronic injections of morphine produced reward-tolerance behaviors that coincided with DA neuron hyperexcitability, decreased DA release in the NAc, and DA soma size reductions (Mazei-Robison et al., 2011; Sklair-Tavron, 1996). Therefore, the morphological changes are relevant to physiological and neurochemical changes that occur in this circuit. The dose of morphine (5.6 mg/kg, 5 days) that produced TH-soma shrinkage in the pVTA in the current study did not produce a significant CPP effect, suggesting this dose was either disruptive or rats became tolerant to the rewarding effect of this dose of morphine over the 5 days. Morphine produces CPP effects in an inverted “U” shaped fashion such that ultra-low and -high doses induce either weak CPP effects, or none at all (Tzschentke et al., 2002). The 3.2 mg/kg dose of morphine produced a robust CPP effect suggesting that this dose is necessary for production of CPP, while 5.6 mg/kg may be the threshold for DA soma shrinkage. The 1.8 mg/kg dose of morphine was sufficient to produce LS effects, but neither CPP nor TH+ soma shrinkage. While morphine produced reward effects ranging from LS to CPP and DA soma shrinkage, EM analog 4 was inactive in all of these models.

In conclusion, at a range of doses producing submaximal, maximal, and supramaximal antinociception, EM analog 4 (1.8 – 5.6 mg/kg, i.v.) did not produce CPP or LS effects. Morphine produced robust CPP and LS effects, consistent with high abuse
liability. Subsequent IHC analysis confirmed reports from human studies showing morphine reduces the size of DA neurons in the pVTA (Mazei-Robison et al., 2011). However, EM analog 4 did not produce any alterations in DA cell morphology in this region. Consistent with the low abuse liability of EM analog 4, these results suggest that chronic exposure to antinociceptive doses of EM analog 4 produced no morphological changes in DA and did not induce reward behaviors. The uncoupling of reward from the analgesic effects of a μ-OR agonist suggests potential for a major improvement in pain therapy.
Chapter 3: Escalation of intravenous self-administration of morphine, but not EM analogs

INTRODUCTION

The absence of conditioned place preference and locomotor sensitization to EM analogs strengthened the argument that the analogs have low abuse potential and do not produce aversive effects. The analogs were next screened in a long-term intravenous self-administration (SA) model to directly measure drug consumption. SA experiments are often conducted with short-term access periods ranging from 1-3 hours, however a long-term access paradigm was chosen in this experiment to maximize the possibility that the EM analogs would be self-administered, and to mimic human drug-taking behavior. Opioids such as morphine, fentanyl, heroin, and oxycodone are self-administered significantly more during long- versus short-term access paradigms (Wade et al, 2015). Here rats were given long access (12 hours/day) to self-administer a range of physiologically comparable doses of morphine or EM analogs. Intravenous SA of drugs of abuse in rodents correlated with over 90% accuracy to human reports of positive subjective effects (O'Connor et al, 2011), so this experiment directly compares morphine and EM analogs in a clinically relevant model.

Two major considerations were addressed in the following experiments: 1. Whether rats will escalate lever pressings to obtain the same dose across sessions and 2. Whether rats will escalate lever pressings to obtain more drug from lower doses across
sessions. The first consideration was addressed by gradually increasing the fixed ratio (FR) requirement from FR1 to FR5 to obtain the same dose, and the second by gradually lowering the available dose across sessions using a constant FR requirement set to FR1. Rats would likely expend more energy to obtain the same dose of a rewarding drug across sessions, and, likewise, escalate responding when the available dose is decreased (Koob and LeMoal, 2006). Conversely, a drug that does not prompt escalated lever responding when required to obtain infusions does not support SA and likely has low abuse potential.

METHODS

Subjects: Rats were catheterized for i.v. infusions as described in Chapter 2. All studies were approved by the Tulane IACUC and adhered to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Self-administration: All SA studies were carried out in 8 standard operant chambers (MED Associates, St. Albans, VT). A light was located above the active lever indicating drug availability and pressings on the inactive lever were recorded but resulted in no scheduled consequence. Tygon tubing connected to a syringe and pump system delivered drug (or vehicle) i.v. through a swivel and leash system that allowed free movement inside the chamber. In 12h/day fixed ratio studies, SA sessions were conducted for 7 days under a gradually escalating fixed ratio (FR) design. The requirement to obtain doses of morphine or analogs (0 - 3 mg/kg/infusion) was increased from FR1 to FR2, FR3, and FR5 on days 1, 3, 5, and 7, respectively. Rats were allowed access to only one dose of drug throughout this experiment and a maximum of 100 infusions was allowed per 12h.
In 12h/day variable dose studies, SA sessions were conducted using a descending dose paradigm in which 0.75, 0.3, 0.1, and 0 mg/kg/infusion of morphine or analogs were available on days 1-2, 3-4, 5-7, and 8-10, respectively.

Data analysis: Data were analyzed by 1-way ANOVA with Neuman-Keuls post hoc comparisons, or 2-way ANOVA (session x drug) with Bonferroni post hoc comparisons when appropriate.

RESULTS

**Long-access self administration.** Overall, EM analogs did not support SA under a long-term (12h per day) access paradigm. SA tests were conducted over 7 days in which rats self-administered morphine, analogs, or an equivalent volume of saline (Figure 3.1). Fixed ratio (FR) infusion requirements gradually increased from FR1 to FR5 over 7 sessions for each group. Lever pressings for doses of morphine, but not any dose of analog 4, increased concurrently with elevated FR requirements. Rats made significantly fewer self-administrations of EM analog 4 than morphine during sessions 5-7, the sessions requiring the greatest amount of effort to obtain the drug ($F[6, 42] = 5.395, p < 0.001$). Morphine was steadily self-administered across the 12hour session ($F[6, 504] = 45.41, p < 0.0001$, Figure 3.2), while EM analogs were no different from controls. Morphine reduced body weight (BW) gain in rats that had unrestricted access to food when comparing session 7 to session 1 ($F[3, 29] = 7.672, p= 0.0006$), suggesting morphine SA reduced appetite. In contrast, EM analog 4 did not impair BW gain ($F[3, 25]= 2.238, p= n.s.$).
Figure 3.1 Escalation of self-administration of morphine, but not EM analog 4, during 12h sessions. a-f. Rats were given access to morphine or analogs for 12h/day for 7 days and the fixed ratio (FR) requirement to obtain infusion was increased on days 1, 3, 5 and 7 to FR1, 2, 3, and 5, respectively. Compared to the inactive lever, morphine was self-administered at a range of doses (note the y-axis scale differences), while EM analog 4 was not. g. Active lever pressing during the FR 3 – 5 schedule from the final 3 sessions were averaged for vehicle, morphine, or analog 4 to compare work-load expenditure. h-i. In the final 3 sessions, infusion rates and intake (mg/kg) per 12h show that morphine, but not EM analog 4, was readily self-administered. +++p< 0.001 compared to vehicle; #, ##p< 0.05, <0.01 compared to the inactive lever; and **p< 0.01 compared to morphine.
Figure 3.2 Average hourly infusion rates indicate morphine was regularly self-administered across the 12h sessions, while analog 4 was no different from controls at any time point. Infusions rates across all sessions were averaged at each hourly time point. a. Morphine was self-administered most readily at 0.75 and 1 mg/kg/infusion. b. Analog 4 was not self-administered more than saline at any time point. c. When comparing the difference in body weight (BW) between the first session and the final session, analog 4 did not impair BW gain compared to controls, while morphine reduced BW gain.

*, **, *** p < 0.05, 0.01, 0.001 compared to saline.

Self-administration dose response. In the variable dose study, self-administrations of morphine increased ($F[1, 12] = 12.87, p < 0.01$) as the available dose was decreased, but pressings for EM analogs were no different from controls (analog 1: $F[1, 8] = 0.4082, p = n.s.$; analog 2: $F[1, 11] = 0.9523, p = n.s.$; analog 4: $F[1, 8] = 3.425, p = n.s.$, Figure 3.3). This indicated that rats were willing to work harder for morphine
infusions when required, but not for the analogs, under two variants of long-term SA conditions.

**Figure 3.3** Self administration (SA) of morphine, but not EM analogs, under a variable dose 12h access paradigm. Descending doses of morphine or analogs were available on the active lever while pressings on the inactive lever resulted in no consequences. **a.** As the available dose of morphine decreased, rats elevated active lever responding for lower doses that do not alone support SA. Rats did not increase responding for any dose of EM analogs. **b.** Inactive lever pressings for morphine or analogs. Active lever presses for morphine, but not analogs, were significantly greater than presses on the respective inactive lever. **c.** Summary of average lever responses by averaging lever pressings for each dose. While rats increased responding for morphine as the dose was lowered, pressings for EM analogs were no different from vehicle. +, ++, +++, $p<0.05$, 0.01, and 0.001 compared to vehicle, #, ## $p<0.05$ and 0.01 compared to the respective inactive lever.
DISCUSSION

This experiment found that under two paradigms of long-term self-administration (SA), rats do not escalate responding for EM analogs. When given access to the same dose across sessions and prompted by elevating fixed ratio requirements, rats with access to morphine significantly escalated active lever responding compared to the inactive lever. By contrast, rats given EM analog 4 did not escalate responding compared to the inactive lever or to rats given vehicle (Figure 3.1). Hourly infusion rates indicated that morphine was consistently and, in some cases, compulsively self-administered across the 12 hour sessions (Figure 3.2) as evidenced by several rats that lever pressed for morphine until the maximum infusion limit (100/12h) was met. Similarly, as the available dose decreased across sessions, rats escalated responding for a dose of morphine that does not produce SA (Figure 3.3). This suggests that prior experience with morphine, but not EM analogs, prompted increased lever responding to obtain infusions. Therefore, in both same-dose and variable-dose access paradigms, EM analogs were not self-administered under long-term access conditions.

Available doses of EM analogs during the SA sessions were based on several considerations: 1. A dose that was inactive on the standard tail flick (TF) test (e.g. 0.75 mg/kg); 2. A dose that produced sub-maximal TF antinociception (1 mg/kg), and 3. A dose that produced maximal antinociception (3 mg/kg) upon injection. Each rat had no prior drug exposure or self-administration experience prior to testing. Morphine was used as a positive control in this experiment because it has repeatedly been shown to be self-administered under a variety of SA paradigms (Doherty et al, 2009; Doherty et al, 2012; Kenny et al, 2006; Martin et al, 2007). When doses of EM analog 4 remained constant
throughout the SA sessions, rats did not escalate responding. Thus, EM analog 4 does not produce the required incentive for rats to expend energy for self-administration (e.g., high FR responding or escalated responding for low doses), suggesting low abuse potential. Consistent with the SA data, EM analogs did not impair BW gain while morphine reduced BW gain when comparing the final session to the first session (Figure 3.2c). Hepburn and co-workers noted that rats dependent on morphine lost significantly more weight after naloxone-precipitated withdrawal, and this was prevented by the δ-OR antagonist naltrindole (Hepburn et al., 1997). Since EM analog 4 shows δ-OR antagonist efficacy (Zadina et al., in preparation), this may have protected against BW loss.

One aspect of reinforcement infrequently tested in SA models is an escalation in SA responding for lower doses. Koob and LeMoal (2006) have suggested that rats trained to self-administer a rewarding dose of morphine will elevate lever-responding when the available dose decreases. Lower doses prompt the rat to increase responding because the lower doses likely do not produce the preferred hedonic state received during previous sessions (Koob et al., 2006). Here rats increased active lever responding for morphine when the available dose was gradually lowered to 0.1 mg/kg/infusion which does not produce SA or antinociception. EM analogs were tested in this variable dose model to determine if prior experience with the analogs would prompt SA of lower doses, however no differences were found when comparing active vs. inactive levers or compared to rats given vehicle. Overall, this data suggests the analogs do not support SA.

Most opioids are readily self-administered by rats and these studies reliably predict human drug-liking and drug-wanting (O'Connor et al., 2011). The absence of SA for EM analogs is consistent with the lack of CPP and LS induction. Since EM analogs
did not significantly alter locomotor behavior, it is unlikely that disruptive effects prevented SA. Since EM analogs did not induce contextual reward as measured by CPP, a lack of escalation in the long term SA models is consistent with this data. Commonly used opioids such as fentanyl, heroin, oxycodone, tramadol, and methadone are readily self-administered during short- or long-term access conditions. Since it appears that the analogs have low abuse liability in a variety of preclinical models, it is interesting to compare the profiles of the analogs to other currently available opioids reported to have reduced abuse liability.

Tramadol, methadone, and buprenorphine are clinically used synthetic opioids for moderate to severe pain and, in the case of methadone and buprenorphine, for opioid addiction maintenance therapy. These µ-OR agonists have each been reported to have reduced side effects through distinct mechanisms. In addition to µ-OR agonist effects, Tramadol’s (+) enantiomer is a norepinephrine/serotonin reuptake inhibitor which could alleviate depression symptoms often co-morbid with pain (Driessen and Reimann, 1992). However, tramadol shows potential for abuse in dependent clinical populations (Cicero et al, 1999). These reports are consistent with preclinical literature showing mixed results in reward models; tramadol induces CPP, can suppress locomotion, does not induce LS (Tzschentke et al, 2002), but rats trained with remifentanil will self-administer tramadol (O'Connor and Mead, 2010). While preclinical reports about tramadol are largely consistent with clinical evaluations, this is not the case with buprenorphine.

Buprenorphine is considered a weak opioid agonist, having activity at µ-ORs, δ-ORs, κ-ORs, but not NOPs. Similarly to tramadol, buprenorphine is reported to provide safe and effective analgesia in non-dependent individuals, but there is evidence that
buprenorphine produces reinforcing effects in dependent heroin users (Comer et al., 2010). Preclinical models indicate buprenorphine does not support SA under long-term access (Wade et al., 2015). Buprenorphine induces CPP and LS, especially when proper training is administered to account for the long half-life of the compound (Tzschentke, 2004). Despite these preclinical findings, buprenorphine is widely used today although it remains debatable whether the good effects (e.g., the analgesia and opiate substitution) outweigh the bad. Likewise, methadone produces CPP (Steinpreis et al., 1996) and SA under short-term access (Martin et al., 2007) so currently there are no available drugs that substitute for morphine that do not produce reward behaviors in rodent models. Overall, the rank order of most reinforcing to least reinforcing opioids in the SA model is fentanyl > heroin > morphine > oxycodone > tramadol > buprenorphine > methadone. However, it is important to note tramadol and methadone have not been tested in the long-term access SA model and both produce CPP effects.

Relative to preclinical reports on these synthetic opioids, the peptide based EM analogs appear to be ideal analgesics since they do not induce three reliable indicators of reward: SA, CPP, or LS. Therefore, these preclinical findings indicate the analogs would have low abuse potential in humans. EM analogs did not support SA under highly sensitive long-term access conditions. Rats worked harder to obtain infusions of morphine, but not EM analogs, under same-dose and variable-dose conditions. EM analog 4 was not self-administered at a range of doses, even doses that produced antinociception on the tail flick test. Drug consumption during SA directly reflects the rewarding effects of a drug of abuse. Data provided here suggest an absence of rewarding
effects for EM analogs during long-term SA paradigms. Overall, EM analogs could provide substantially improved pain therapy with reduced reinforcement.
Chapter 4: EM analogs penetrate the blood-brain barrier (BBB) to produce mu-selective antinociceptive effects

INTRODUCTION

Since the CPP and SA tests indicated reduced abuse liability of EM analogs, one interpretation is that they do not penetrate the BBB to produce centrally-mediated reward effects. While the long duration of antinociception induced by EM analogs makes this possibility unlikely, an experiment was devised to test the BBB penetration of the analogs. Studies have established that systemic naloxone, but not delta or kappa antagonists, blocks antinociception from the analogs, indicating µ-ORs modulate their antinociception (Zadina et al, in preparation). This was further supported by antagonism with the µ-OR selective antagonist βFNA in the tail flick test. However, it is unclear whether the analogs predominantly activate peripheral or central opioid receptors populations to elicit antinociception. Naloxone methiodide (Nlx-M) is a variant of naloxone hydrochloride. Nlx-M antagonizes central opioid receptors after intracerebroventricular (i.c.v.) administration and, if injected peripherally, only antagonizes peripheral opioid receptor populations (Al-Khrasani et al, 2012; He et al, 2009). Therefore, Nlx-M was used to study central penetration effects of the EM analogs. In addition, a hot plate test, which requires central mediation of antinociceptive responses, was used to assess central penetration and reversal by βFNA.

METHODS
Animals: Male CD1 albino mice and male Sprague Dawley rats were purchased from Charles River. Rats were tested at approximately 275-350g and mice were tested at approximately 25-35g. All animals were habituated to the colony room for at least 5 days prior to behavioral testing and room temperature was maintained at 22°C. Mice were housed 4-5 per cage prior to testing; rats were housed 2-3 per cage before surgery and single housed after. Rats were catheterized in the jugular vein as described in chapter 2. All animals were housed under a 12:12 light/dark cycle (lights on at 07:00). Experiments were conducted during the light cycle. All experimental procedures were approved by the Tulane Institutional Animal Care and Use Committee.

Intracerebroventricular (i.c.v.) injection technique: Since only a single i.c.v. injection was required for these experiments, we used a direct i.c.v. injection method. Rats were placed in a stereotaxic apparatus under a mixture of isoflurane/oxygen (4-5% induction, and 1.5-2.5% for the remainder of the injections) anesthetic. Coordinates for the right lateral ventricle were determined using the landmark bregma: 1.5 mm lateral, 0.7 mm posterior, and 3.5 mm ventral to bregma. Drug or vehicle (saline) infusions were made using a 5ul Hamilton syringe (Fisher Scientific, Pittsburgh, PA) in a volume of 5ul. The injection was made over 1 minute and the syringe was held in place for 1 additional minute to ensure adequate diffusion.

Antinociception assays: The tail flick (TF) test was used to determine antinociception by measuring latency to withdraw the tail from a heat source applied by a TF apparatus (IITC, Woodland Hills, CA) that captured time (sec) to withdraw. Baseline latencies were approximately 3-4 sec and a 9 sec cut-off time was used to prevent tissue damage. Three
baseline TF latencies were taken prior to drug injection, and at 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 mins post injection, or until %MPE fell below 20%. Data were converted into maximum possible effect (MPE) values by the following formula:

\[
[(\text{latency}-\text{baseline latency})/(9-\text{baseline latency})]\times100.
\]

Bolus i.v. doses were chosen based on pilot data showing immediate and maximal (100% MPE) antinociception for morphine (3.2 mg/kg) and analogs (3.2 mg/kg). For the antagonist study, the dose of Nlx-M (10 ug/5ul, i.c.v.) was chosen based on literature reports (Al-Khrasani et al, 2012) showing full antagonism of systemic morphine at this dose. TF data were converted to area under the curve (AUC) values to factor both the magnitude and duration of antinociception and to facilitate statistical comparisons. Intravenous injections of the analogs elicited similar or longer duration of antinociception than morphine.

The hot plate (HP [IITC, Woodland Hills, CA]) test was used to measure antinociception in mice. The HP apparatus was set to 52.5°C, a temperature that elicits pain responses after approximately 7-9 sec. Mice were removed from the HP after a maximum of 30 sec. Beta-funaltrexamine (βFNA) or saline was injected 24 hours prior to HP testing. Three baseline HP latencies to rapidly lift, lick, or shake the hind paws were taken prior to drug injection. Mice were then injected with EM analog 4 (0 – 130 mg/kg s.c.) and tested 30, 45, 60, 90, 120, 180, and 240 mins after injection. Data were converted into maximum possible effect (MPE) values by the following formula:

\[
[(\text{latency}-\text{baseline latency})/(30-\text{baseline latency})]\times100.
\]

Data analysis: Data were analyzed by one-way ANOVA with Neuman Keuls post hoc comparisons.
RESULTS

**BBB penetration experiment.** Rats were pretreated with either vehicle or naloxone methiodide (Nlx-M [10ug, i.c.v.]) 20 minutes prior to intravenous injections of morphine (3.2 mg/kg), EM analogs (3.2 mg/kg), or vehicle and tested for tail flick (TF) response (Figure 4.1). Baseline TF responses did not differ between groups. When comparing vehicle, morphine, and EM analogs with and without Nlx-M, the ANOVA showed a significant effect ($F[11, 45]= 23.01, p< 0.0001$). Overall, EM analogs produced equal or greater AUC values than morphine (++, +++p< 0.01, 0.001 compared to vehicle). Antinociception from morphine and all analogs were attenuated by i.c.v. Nlx-M pretreatment (*,**,***p<0.05, 0.01, 0.001 compared to respective non-antagonist injected group). EM analog 4 produced the longest duration of TF antinociception and was strongly blocked by Nlx-M, so this analog was chosen for the βFNA reversal experiment.
Figure 4.1 Central penetration of EM analogs. a-e. Timecourse for the antinociceptive effects of i.v. EM analogs or morphine 20 mins after central opioid receptor blockade with naloxone methiodide (Nlx-M; 10µg, i.c.v.). f. Area under the curve (AUC) values calculated from TF response latencies after i.v. vehicle, morphine, or analogs and i.c.v. injection of Nlx-M or vehicle. Nlx-M attenuated the antinociceptive effects of morphine and all analogs. ++, +++ p< 0.01, 0.001 compared to vehicle, *, **, *** p< 0.05, 0.01, 0.001 compared to respective non-antagonist injected group. Note the x-axis scale differences with the analogs generally producing longer duration of antinociception than morphine.

**Hot plate (HP) experiment.** EM analog 4 increased reaction latencies on the HP test with maximal antinociception (>80% MPE) at a dose of 130 mg/kg (figure 4.2). This dose was chosen for antagonism by βFNA. ANOVA showed a significant difference
between groups given vehicle or EM analog 4 with and without βFNA ($F[5, 31] = 14.74, p < 0.0001$). βFNA was found to have no antinociceptive properties alone (figure 2b). Twenty-four hour pretreatment with βFNA (40 mg/kg, s.c.) blocked the antinociceptive effects of EM analog 4 ($p < 0.001$).

![Figure 4.2](image)

**Figure 4.2** Hot plate (HP) antinociception is produced by EM analog 4 and attenuated by a selective µ-OR antagonist. **a.** HP latencies to lick or shake the paw were measured at regular intervals and converted to %MPE. **b.** Area under the curve (AUC) values for analog 4 show dose-dependent HP antinociception ($+ p < 0.05$, $+++ p < 0.001$, compared to vehicle) that was attenuated by the µ-OR selective antagonist βFNA (40 mg/kg, s.c.). $+$, $+++ p < 0.05$, 0.001 compared to vehicle; $*** p < 0.001$ compared to analog 4 alone.

**DISCUSSION**

This experiment found that EM analogs likely penetrate the BBB since intracerebroventricular (i.c.v.) injection of naloxone methiodide (Nlx-M) attenuated the antinociceptive effects of all EM analogs (Figure 4.1). If any Nlx-M diffused to the periphery, the 10µg amount that was injected is over 2 orders of magnitude below a standard peripheral dose of 10 mg/kg, so this possibility is unlikely. Recent preliminary data suggests that peripheral co-administration of Nlx-M with the analogs had no effect
on antinociception, so central mechanisms likely modulate antinociception from the analogs. There were antinociceptive differences between individual analogs and morphine on the TF test. Analogs 1 and 4 produced significantly greater area under the curve (AUC) TF antinociception than morphine, while analogs 2 and 3 produced similar AUC values compared to morphine at the same dose. Furthermore, Nlx-M pretreatment antagonized morphine and the analogs differentially. Nlx-M strongly antagonized the antinociception of morphine in addition to analogs 3 and 4, while analogs 1 and 2 were more modestly reduced. This indicates that analogs 3 and 4 may be better at penetrating the BBB than analogs 1 and 2. Since analog 4 showed the greatest duration of antinociception and most favorable side effect profile, this data supports the choice of analog 4 as a lead compound. Overall, central opioid receptors appear necessary for maximal antinociceptive effects of EM analogs.

Factors affecting the ability of a compound to cross the BBB include size, charge, lipid/water solubility, whether it is a substrate for a transporter, it’s conformation (linear vs. cyclic), and its amphipathic properties. Future studies are planned to develop an LC-MS protocol for determining pharmacokinetic parameters that include direct measures of BBB penetration. The present data provide evidence that all 4 analogs are able to penetrate the BBB in an *in vivo* model.

The EM1 and EM2 parent peptides have the highest selectivity for the µ-OR compared to any other endogenous opioid (Zadina *et al.*, 1997). This experiment showed that µ-ORs are required for the HP antinociceptive effects of EM analog 4 because βFNA, the highly selective µ-OR antagonist, attenuated these effects. βFNA also partially blocks the antinociceptive effects of other high efficacy µ-OR agonists including fentanyl.
and DAMGO. βFNA abolishes the effects of low efficacy agonists such as buprenorphine, suggesting that the extent of βFNA antagonism depends on opioid efficacy. Negus and colleagues showed that βFNA shifted the dose response curve of fentanyl to the right rather than decreasing the maximum effect. This is consistent with the concept that high efficacy agonists access a spare receptor reserve pool in order to produce residual effects after an irreversible antagonist injection (Negus et al, 1993). EM analog 4 appears to have high efficacy and selectivity for μ-ORs, so this compound could more effectively treat pain disorders due to its markedly improved analgesia/side effect ratio and BBB penetration.

In conclusion, EM analogs require centrally located opioid receptors to produce maximal antinociception. Central administration of Nlx-M (i.c.v.) weakened the antinociceptive effects of EM analogs, suggesting penetration of the BBB. EM analog 4 appears to produce high efficacy μ-OR agonist effects since the selective μ-OR antagonist βFNA partially blocked the antinociceptive effects. Since analog 4 likely penetrates the BBB to produce antinociception and μ-OR antagonism reduced the antinociceptive effects on the HP test, analog 4 has an advantageous profile to treat CNS-related pain disorders due to its long duration of antinociception and reduced side effects.
Chapter 5: Differential tolerance and inflammatory effects induced by EM analogs compared to morphine

INTRODUCTION

Chronic opioid therapy creates difficult problems for doctors who must weigh the risk of inducing tolerance and dependence with effectively managing pain. Ideally a pain reliever should produce long lasting analgesic effects and produce less tolerance than the gold-standard pain reliever, morphine. Tolerance can be modeled in rats by implanting chronic catheters in the intrathecal (i.t.) space of the spinal cord. A minipump attached to the i.t. catheter restricts chronic infusions to the lumbar spinal cord. Rats begin to develop analgesic tolerance to chronic infusions of i.t. morphine after approximately 2-3 days (Stevens and Yaksh, 1989a). High potency μ-OR agonists such as DAMGO promote substantially less tolerance than equivalently antinociceptive doses of morphine in this model (Stevens et al, 1989b). A drug that promoted less tolerance and abuse liability would likely provide more efficacious pain therapy. Therefore, part 1 of this experiment evaluates tolerance assessed after chronic infusions of morphine or EM analogs.

Part 2 will attempt to link the chronic effects of morphine and EM analogs to ongoing work suggesting that immune reactivity contributes to the development of tolerance. Several groups have proposed an immune reactivity hypothesis for the development of tolerance to the analgesic effects of opioids (Chen et al, 2012; Hutchinson et al, 2011; Narita et al, 2006; Wang et al, 2010; Zhou et al, 2010). Glial
cells such as astrocytes and microglia survey the surrounding environment for potentially dangerous foreign substances and emit cytokines and chemokines that promote inflammation. Proinflammatory cytokines also activate nociceptive neurons and can contribute to the greater requirement for opioids to alleviate pain (i.e., tolerance). One proposed mechanism by which microglia and astrocytes promote tolerance to the analgesic effects of morphine is via interaction at specific binding sites called toll-like receptors [TLRs (Hutchinson et al., 2012; Hutchinson et al., 2010b)]. TLR signaling has been shown to contribute to tolerance from morphine through a phosphorylated-p38 (pp38)-dependent mechanism (Hutchinson et al., 2010a). While efforts are underway to develop TLR4 inhibitors as adjunctive therapy with morphine to reduce tolerance (Hutchinson et al., 2009), an opioid that does not engage immune reactivity may be more effective for the treatment of chronic pain. Therefore, following the tolerance paradigm described above, microglial and astrocytic activation markers were compared in rats chronically infused with morphine, EM analogs, or vehicle.

A second proposed mechanism is CGRP-dependent neuron-glia signaling that has been suggested to promote tolerance through activation of microglia and astrocytes (Horvath et al., 2010b; Wang et al., 2010). For example, the CGRP antagonist BIBN4096BS prevented the development of tolerance to morphine by avoiding the pp38 mechanism also activated by TLR signaling (Wang et al., 2010). CGRP activation of receptors located on microglia, astrocytes, and neurons promotes a cascade of cellular events that collaboratively increase IL-6, IL-1β, and NMDA receptor components from these cell types, respectively. The model proposes that CGRP upregulation is a key first step that mobilizes proinflammatory signaling molecules to promote tolerance to
morphine. In addition to blocking tolerance to morphine, BIBN4096BS alleviated inflammatory pain induced by CFA injections (Hirsch et al, 2013), suggesting CGRP activation and de-activation has clinical relevance for both pain relief and tolerance. Therefore, CGRP expression was compared in rats chronically infused with morphine, EM analogs, or vehicle.

Finally, morphine promotes activation of purinergic (P2X) signaling by microglia that may stimulate glial cell activation to promote tolerance. A P2X7 receptor agonist, Brilliant Blue G (BBG) was shown to attenuate tolerance produced by morphine (Chen et al, 2012). Furthermore, morphine upregulated P2X7 receptors along with microglial signaling markers Iba1 and OX-42 (Zhou et al, 2010). P2X7 receptors likely activated pp38, the common inflammatory pathway induced by TLR and CGRP activation that increases cytokine expression and induces tolerance to morphine. It is unknown whether EM analogs engage any of these pathways after chronic infusions, however opioid agonists that do not engage these proinflammatory circuits may translate to less tolerance after chronic infusions and more effective alleviation of pain.

METHODS

Animals: Male Sprague-Dawley rats (250-350g, Charles River, Wilmington, MA) were housed similarly to previous experiments and procedures were approved by the Tulane Institutional Animal Care and Use Committee and conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Chronic intrathecal (i.t.) surgery: The Yaksh (Stevens et al, 1989a) method was used to implant rats with catheters made with PE-8 (0.008” I.D.) tubing inserted into the rostral
end of the subarachnoid space. The catheter tip was slowly moved to the L4 region, and
the rostral exit port was secured with sutures. Rats were allowed to recover for at least 7
days prior to testing. Lidocaine (20µl at 2%) was injected through the i.t. catheter to test
placement of the catheter tip. Only rats that displayed temporary (1-2 min) paralysis were
used for the study.

Antinociception: The standard tail flick (TF) was used to measure the latency to
withdraw the tail from a heat source (IITC, Woodland Hills, CA). Baseline TF latencies
were 3-4 sec with a cutoff time of 9 sec to prevent tissue damage. Maximum possible
effect (MPE) was determined as [(latency-baseline latency)/(9-baseline latency)]*100.
Acute intrathecal (i.t.) injections were assessed using a cumulative dosing model in which
doses were increased in ¼ log increments with injections every 20 min followed 15
minutes later by TF tests. Immediately after acute tests, osmotic minipumps (Alzet model
2001, Durect Corp,Cupertino, CA), filled with vehicle, morphine, or analog and primed
in 0.9% saline at 37°C for 16h, were implanted s.c. and connected to the i.t. catheter. The
pumps delivered 8x the ED50/hr (2 ug/hr morphine, 0.056ug- 0.075ug/hr analog) for 7
days. A second dose-response curve was generated on day 7. Acute and chronic ED50
values were generated and the shift in ED50 after chronic infusions provided an index of
relative tolerance.

Immunohistochemistry: Animals were deeply anesthetized with ketamine/xylazine (85/10
mg/kg) and perfused transcardially with 0.1M PBS followed by 4% paraformaldehyde.
Spinal cords were post-fixed overnight at 4°C, cryoprotected in 30% sucrose/0.1M PBS
for 48 hr, and sectioned on a freezing microtome at 40µm. After 2 washes in PBS and
blocking with 5% normal goat serum/0.3% Triton X-100, sections were incubated in
primary antibody; GFAP (1:1000, ab7779, Abcam, Cambridge, MA), Iba1 (1:1000, #019-19741, Wako, Richmond, VA), pp38 (1:100, #4511, Cell Signaling Technology, Danvers, MA), or CGRP (1:1000, T-4032, Peninsula Labs, San Carlos, CA) for 24h at 4°C on a slow rocker. The tissue was then washed twice, re-blocked, incubated in donkey anti-rabbit secondary antibody conjugated to Alexa488 (1:500 for GFAP, Iba1, and CGRP; 1:200 for pp38, #A21206 Life Technologies, Grand Island, NY) for 2hrs at RT, washed, and slide-mounted with Prolong Gold (Life Technologies). GFAP- and Iba1-immunoreactivities in lamina I-V of dorsal horn segments were quantified on a Nikon microscope with a Hamamatsu camera and NIH ImageJ software. Images containing lamina I-II of the spinal dorsal horn were analyzed for P2X7, CGRP and OX-42 integrated density using ImageJ (Smith et al., 2001). A blinded observer determined integrated density by thresholding the images using the default ImageJ algorithm to reduce background and only include positively stained cells. Integrated density in the region of interest (ROI) is equal to the product of area and mean gray value. The mean gray value represents the sum of the intensity values/number of pixels for all pixels above the threshold in the ROI. This method controls for differences in background between slices and subjects. For quantification of pp38, an observer blinded to treatment manually counted punctate immunoreactive cells. For co-labeling experiments, primary antibody against P2X7 receptors (P2X7R; 1:100, #APR-008, Alamone Labs, Jerusalem, Israel) was incubated with OX-42 overnight. Tissue was washed and re-blocked as described above, and finally incubated with appropriate secondary antibodies, Alexa488 (1:500) and Alexa594 (1:500), before washing and mounting. Quantification of P2X7R and OX-42 co-labeling was performed using Nikon projection images constructed from 1µm thick
image stacks. The number of OX-42 positive cells and P2X7R/OX-42 co-labeled cells were counted to determine percent co-labeling. A total of 5-6 rats per group and 4-6 slices/rat were quantified for all experiments. Representative confocal images were generated on a Leica SP2 AOBS microscope.

Data analysis: Data were analyzed by ANOVA followed by Newman-Keuls post-tests, and ED$_{50}$’s were generated by non-linear regression using Prism (GraphPad, San Diego, CA).

RESULTS

**Reduced tolerance to chronic EM analogs.** Rats implanted with chronic indwelling i.t. catheters were cumulatively injected with i.t. morphine or EM analogs before and after chronic minipump infusions of morphine or EM analogs. The acute potency of EM analogs was about 30 times higher than morphine (ED$_{50}$s: analog 1 = 0.0084; analog 2 = 0.0082; analog 3 = 0.0074; analog 4 = 0.0081; versus morphine = 0.2055 µg). Dose-response curves generated after chronic minipump infusions of morphine (ED$_{50}$ = 7.748 µg) or EM analogs (ED$_{50}$s: analog 1 = 0.1116; analog 2 = 0.0867; analog 3 = 0.1194; analog 4 = 0.1222 µg) showed that morphine produced a 37-fold shift in tolerance, while EM analogs averaged only a 13-fold shift (Figure 5.1).
Figure 5.1 Reduced tolerance and lack of glial cell activation by EM analogs relative to morphine. 

a. Dose response curves were generated by TF latencies prior to- (pre, open symbols) and after- (post, closed symbols) 7-days of chronic minipump infusion of i.t. morphine or EM analogs (pump dose= 8x acute ED50s). ED50 values are presented on the right along with the shift in relative tolerance after chronic infusions, showing the analogs produced substantially less tolerance than morphine. 

b. Spinal cord tissue from these rats was assessed for glial cell activation. Representative photomicrographs were generated for vehicle, morphine, or analog 4 infused rats by a confocal microscope (scale bar= 100um, inset= 50um). 

c. Glial cell activation was quantified by integrated density threshold adjustment for GFAP and Iba1 or by counting the number of pp38 positive cells. All three markers were significantly elevated by morphine, but not EM analogs. ++, ++++, p< 0.01, <0.0001 compared to vehicle; **, ****, p< 0.01, <0.0001 compared to morphine.
Glial cell CGRP and P2X7 receptor activation by morphine, but not EM analogs. Dorsal spinal cord sections from rats receiving chronic infusions of morphine or EM analog showed that morphine, but not analogs, increased glial cell activation markers (Figure 5.1), CGRP (Figure 5.2), and P2X7 co-labeling (Figure 5.3). Morphine increased the astrocytic marker GFAP ($F[5, 25]= 3.612, p = 0.0135$), the microglial marker Iba1 ($F[5, 24] = 4.705, p = 0.0039$), and the microglial signaling kinase pp38 ($F[5, 25]= 16.83, p < 0.0001$) compared to controls. However, rats infused with EM analogs showed no difference from controls. Furthermore, morphine increased CGRP expression ($F[5, 32]= 4.504, p = 0.0032$), and increased co-labeling of P2X7 receptors with OX-42 labeled microglia ($F[2, 12]= 15.61, p= 0.0005$) compared to vehicle, while EM analogs were no different from controls.

**Figure 5.2** Chronic intrathecal infusions of morphine, but not EM analogs, increases CGRP expression in the dorsal spinal cord. a. Spinal cord area of analysis. b. Integrated density
quantification of CGRP in lamina I-II shows that morphine upregulated CGRP, while EM analogs did not. 

Representative photomicrographs from rats infused with vehicle, morphine, or analog 4 (scale bar=50um).

![Graphs showing OX-42 and P2X7 receptor upregulation](image)

**Figure 5.3** P2X7 receptor upregulation in microglia cells after chronic infusion of morphine, but not EM analog 4. 

a. Chronic morphine strongly upregulated OX-42 microglial cells in lamina I-II. 

b. While rats given chronic morphine showed increased microglial-P2X7 co-labeling, infusions of EM analog 4 did not increase co-labeling compared to vehicle. 

c.. Representative images of P2X7 receptors co-labeled with OX-42 microglial cells were taken from vehicle, morphine, and analog 4 infused rats (scale bar = 20 µm). Morphine infused spinal sections contained microglial cells expressing the activated phagocytic phenotype, but sections...
from vehicle and EM analog 4 infused rats did not. These activated microglial cells often co-labeled with P2X7 receptors (~21% in morphine vs. 5% in analog 4 infused rats) suggesting potential ATP-P2X7-neurotrophin-cytokine mediated effects of morphine, but not analog 4. +, ++ \( p < 0.05, < 0.01 \) compared to vehicle; *, ***, \( p < 0.05, < 0.001 \); n= 5-6 rats, 4-6 sections per rat compared to morphine.

DISCUSSION

This study demonstrated that chronic EM analogs produced substantially less tolerance than morphine and did not induce glial cell, CGRP, or P2X7 activation. Acute intrathecal (i.t.) injection of EM analogs produced maximal antinociception about 30 times more potently than morphine, indicating the EM analogs are high potency agonists (Figure 5.1). Chronic infusions of morphine produced substantially more tolerance (37-fold) than chronic EM analogs (~13-fold). Following the tolerance protocol, rats were sacrificed and spinal cord sections were assessed for glial cell markers, P2X7, and CGRP. Chronic morphine, but not EM analogs, promoted glial cell activation in the dorsal spinal cord as measured by increased GFAP, a marker for astrocytes, Iba1, OX-42, and pp38, markers for microglia (Figure 5.1 and 5.3). Similarly, chronic morphine increased CGRP expression in the dorsal spinal cord, whereas EM analogs produced no change compared to controls (Figure 5.2). Finally, P2X7 receptors were increased specifically in microglial cells expressing OX-42 from rats that received chronic morphine, but not EM analog 4 (Figure 5.3). Therefore, relative to morphine, the analogs produced less tolerance that was not accompanied by glial cell activation, CGRP upregulation, or microglial-P2X7 signaling.

In this experiment the chronic i.t. infusion dose was normalized to 8 times the acute ED\(_{50}\) of morphine or EM analogs. Since rats were given EM analogs at the same
relative dose as morphine, we conclude that EM analogs produce significantly less tolerance after chronic infusions. This is consistent with studies showing that high potency agonists such as DAMGO and sufentanil promote less tolerance than low potency agonists such as morphine (Stevens et al, 1989b). While the high potency effects of the analogs likely contributed to the reduced tolerance, the lack of glial reactivity, CGRP, and P2X7 signaling may have also contributed.

Several groups have demonstrated that activation of glial cells promotes inflammation and exacerbates tolerance to the antinociceptive effects of morphine. Chronic morphine increases expression of markers for astrocytes and microglia in spinal (Wang et al, 2009) and supra-spinal (Hutchinson et al, 2009) regions. In this study morphine upregulated the astrocytic marker GFAP, and the microglial markers Iba1 and pp38 in the lumbar spinal cord where infusions were made. In the same model, EM analogs did not activate any of these markers indicating an absence of glial reactivity. Since glial cell inhibitors such as minocycline (Hutchinson et al, 2008) and propentophyline (Narita et al, 2006) attenuate tolerance and dependence to morphine, efforts to develop drugs that increase the analgesic effectiveness and reduce tolerance to morphine are underway. However, the development of drugs that do not engage inflammatory circuits while still providing equivalently analgesic effects as morphine could prove more effective. EM analogs appear to meet this goal since they lacked glial cell activation and produced substantially less tolerance.

Efforts to develop compounds that block the CGRP-activating effects of morphine are currently underway (Wang et al, 2009). Chronic morphine induces CGRP release from primary afferent nerve terminals in dorsal root ganglia neurons that synapse in the
dorsal horn. CGRP receptor activation in the dorsal horn increases expression of cytokines such as IL-1β and IL-6 from astrocytes and microglia, respectively. CGRP also acts on neuronal receptors to increase production of NMDA receptor components known to modulate tolerance to morphine (Mendez et al., 2008). Overall, upregulation of CGRP in the dorsal spinal cord results in astrocyte-microglia-neuron crosstalk that exacerbates tolerance to morphine-induced antinociception. Co-administration of CGRP or NMDA antagonists that block tolerance to morphine could indeed improve the effectiveness of morphine, but the development of a single drug that does not activate CGRP would be more ideal for pain therapy. Therefore, the absence of CGRP induction by EM analogs may have contributed to the reduced tolerance.

Recent findings show that ATP-stimulated P2X receptors may counteract the antinociceptive effects of morphine. Spinal P2X7 receptors located on microglial cells were strongly upregulated in morphine-tolerant rats and it was found that siRNA knockdown of P2X7 receptors attenuated tolerance to morphine (Zhou et al., 2010). Furthermore, the cytokine IL-18 was found upregulated in microglial cells in morphine-tolerant rats and IL-18 interference blocked morphine-tolerance (Chen et al., 2012). IL-18 is an important cytokine since it can increase release of D-serine, a modulator of NMDAR activation. NMDAR activation has been linked to morphine-tolerance since NMDAR antagonists block tolerance to morphine. In this experiment, P2X7 receptors strongly co-labeled with microglial cells in rats tolerant to morphine, but co-expression was not found in rats given equi-effective doses of EM analog 4. The lack of P2X7 induction by analog 4 suggests fewer ATP stimulated excitatory effects and less proinflammatory glia-neuron signaling by analog 4.
Overall, EM analogs induced less tolerance that did not promote glial cell activation, CGRP expression, or P2X7 upregulation. This study suggests a potential mechanism for the reduced tolerance and superior antinociceptive effects of EM analogs compared to morphine. Converging evidence from multiple laboratories suggests that glial cells independently, and through neuron-glial cell crosstalk, promote tolerance to morphine. Since the EM analogs were based on naturally occurring endomorphin peptides, the similarity of the analogs to the endogenous peptides may have protected against glial reactivity, CGRP-, and P2X7-dependent inflammation. Since EM analogs do not engage these proinflammatory mediators, this could translate to better clinical pain management with reduced tolerance.
Chapter 6: Drug discrimination effects of EM analogs in morphine-trained rats

INTRODUCTION

EM analogs have clear indications for the treatment of pain conditions and the reduced side effects of the analogs indicate they may also be useful treatment for opioid dependency. Opioid dependency is often treated by other μ-OR agonists such as methadone or buprenorphine since these compounds substitute for heroin. However, these compounds are closely regulated since they have their own propensity for abuse as shown in SA, LS, and CPP models (Martin et al., 2007; Steinpreis et al., 1996; Tzschentke, 2004; Wade et al., 2015). Agonists of the μ-OR with reduced side effects, such as the analogs, may more effectively substitute for heroin or morphine. The drug discrimination (DD) test investigates mechanisms of the subjective effects of psychoactive drugs (Young, 2009) including opioids. A core feature of this experiment is that a drug injection sends a signal to the subject that prompts the necessary responding in order to receive reinforcement such as money for humans or food pellets for mildly food-restricted animals. DD test sessions probe whether experimental compounds of similar class, structure, and/or activity substitute for the training compound. The advantage of DD is that a highly sensitive relationship is established between a training drug and the appropriate response. The ability of experimental drugs (e.g., the analogs) to substitute for a training drug (e.g., morphine), could have implications for substance abuse treatment and opioid maintenance therapy.
Rats readily learn to discriminate morphine from vehicle injections in the DD model, but not peripherally restricted drugs such as loperamide (Gianutsos et al., 1975). This suggests blood-brain barrier (BBB) penetration is a requirement for DD. Opioids can produce disruptive effects on DD food-reinforcement rates which are tested in conjunction with substitution effects. For example, methadone, buprenorphine, and fentanyl all substitute for morphine in the DD model, but also impair responding at doses that substitute (Craft et al., 1999; Vann et al., 2009). A drug that substitutes for morphine with less response rate impairment may have a more favorable profile for the pharmacotherapy of opioid abuse because the subject may have greater functionality while receiving treatment. Ideally, the same compound would not itself promote reward behaviors such as SA or CPP. Currently there are no compounds that meet the rigid criteria of penetrating the BBB to cue morphine-lever responding, but not produce disruptive or rewarding effects.

This experiment tested the hypothesis that EM analogs substitute for morphine in the DD model with less disruptive effects. A drug that does not produce rewarding effects, but substitutes for morphine could translate to more effective opioid maintenance therapy. Easing the transition from an opiate-dependent state to a more manageable clinical maintenance could restore functionality and productivity in this population.

METHODS

Drug Discrimination (DD): Male and female rats were food deprived to approximately 85% of the weight of free feeding cohorts to establish operant responding for 45mg food pellets (Bioserve, Frenchtown, NJ) in standard operant chambers (MED Associates, St.
Albans, VT). As shown in table 6.1, DD consisted of 3 phases (Krivsky et al, 2006). In **phase 1**, rats were trained to lever press for food at a fixed ratio (FR) level 1 (e.g., 1 lever press=1 food pellet) using 2 levers with stimulus lights located above each lever and a food hopper between the levers. Responding on either lever extinguished the light and delivered a food pellet. Once the rats earned 100 food pellets in a 120 min session for 2 consecutive sessions, response requirements increased to FR2, FR5, FR7, and FR9. Once ≥ 20 pellets were earned at FR9, sessions were shortened to 20 mins and response requirements were set at FR10 and the left or right lever reinforced responding on alternate days. Once ≥20 pellets were earned on each lever at FR10, rats started phase 2 of DD training. **Phase 2** sessions consisted of pretreatment with vehicle or morphine (3.2mg/kg, s.c.) 30 mins prior to the 20 min session and food pellets were only available on the vehicle (saline) or morphine lever, as indicated by a light above the lever. Half the rats were trained with morphine on the left lever and vehicle on the right while the other half were trained vice-versa. The daily order of vehicle (V) or morphine (M) injections was set on an unpredictable 4 week schedule: week 1: M, V, V, M, V; week 2, V, M, M, V, M; week 3: V, V, M, M, V; week 4: M, M, V, V, M. When ≥ 20 pellets were earned on both morphine and vehicle days, both stimulus lights were illuminated such that only the injection cued the lever responding. Only responses on the correct lever were reinforced and responses on the incorrect lever reset the response requirement on the correct lever. Criteria to move to **phase 3** were as follows: ≥20 pellets per session, ≥90% injection appropriate lever responding, and ≤9 responses on the incorrect lever before the first food pellet reward. At this point, substitution tests of s.c. morphine were conducted in which a range of morphine doses (0, 0.3, 1, 1.8, 3.2 mg/kg) were administered on
Mondays and Thursdays, where pressings on either lever were reinforced, and training sessions were administered on the intervening days.

After completion of the s.c. dose response for morphine, intravenous (i.v.) catheters were implanted and rats recovered for 5 days as described in Chapter 2. Training sessions continued using the i.v. route with a ¼ log lower dose of morphine (1.8 mg/kg), since the antinociceptive potency of i.v. morphine is slightly higher than that of the s.c. route (South et al, 2009). Two paradigms were utilized to generate substitution curves for the analogs: a cumulative “within” session- and a between session- injection model. For the between session model, substitution curves were generated for morphine and EM analogs 1-4 (0, 0.3, 1, 1.8, 3.2, 5.6 mg/kg, i.v.) in a pseudo-random order on Mondays and Thursdays using the i.v. route; food reinforcement occurred after meeting the FR10 requirement on either lever. Training sessions were administered on the intervening days to ensure accurate responding. Substitution tests only occurred after rats met the criteria for 4 days and met the criteria on the most recent vehicle or morphine training session. During substitution tests, both levers actively delivered food at an FR10 schedule. Some substitution tests were conducted with naloxone (0.1 – 5 mg/kg, i.v.) co-administered with morphine or analogs to block discriminative stimulus responding. The cumulative injection model was conducted within a single session with morphine, analogs, or vehicle (Varner et al, 2013). Doses were increased in ¼ log increments, with injections every 20 min followed 15 minutes later by DD sessions lasting 5 mins each. Doses of morphine or analogs were increased cumulatively by injecting 0.3, 0.7, 0.8, 1.4, and finally 2.4 mg/kg to achieve doses of 0.3, 1, 1.8, 3.2, and 5.6 mg/kg. The four 5-min sessions were identical to the 20 min substitution tests. The advantage of this procedure is
that an entire dose-response is generated in one session. Lastly, a time course for DD responding was conducted using 5 min test sessions beginning 15, 30, 60, 90, and 120 min after i.v. injection of morphine or analog (5.6 mg/kg, i.v.) on the same day. Each rat received a maximum number of 5 substitution tests with morphine and analogs, 2 antagonist tests, and 2 time course experiments.

Data analysis: Data were analyzed by ANOVA followed by Newman-Keuls post-tests, and ED<sub>50</sub>'s were generated by non-linear regression using Prism (GraphPad, San Diego, CA).

<table>
<thead>
<tr>
<th>Phase</th>
<th>Schedule</th>
<th>Lever for Food</th>
<th>Stimulus Light Illuminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Food training</td>
<td>Both levers</td>
<td>Both</td>
</tr>
<tr>
<td>1b</td>
<td>Food training</td>
<td>Only Left or Only Right (FR10)</td>
<td>Only Correct lever</td>
</tr>
<tr>
<td>2a</td>
<td>Morphine s.c. training</td>
<td>Only Morphine or Vehicle lever</td>
<td>Only Correct lever</td>
</tr>
<tr>
<td>2b</td>
<td>Morphine s.c. training</td>
<td>Only Morphine or Vehicle lever</td>
<td>Both</td>
</tr>
<tr>
<td>2c</td>
<td>Morphine s.c. testing</td>
<td>Both levers</td>
<td>Both</td>
</tr>
<tr>
<td>3a</td>
<td>Morphine i.v. training</td>
<td>Only Morphine or Vehicle lever</td>
<td>Both</td>
</tr>
<tr>
<td>3b</td>
<td>Morphine or EM analog i.v. testing</td>
<td>Both levers</td>
<td>Both</td>
</tr>
</tbody>
</table>
**EM analogs substitute for morphine with less disruption in the drug discrimination (DD) model.** The DD effects of EM analogs were studied in rats trained to discriminate s.c. and i.v. infusions of morphine (Table 6.1). After approximately 20 sessions, morphine produced almost 100% response accuracy while vehicle produced only 0.6% morphine-responding in male rats (figure 6.1). This indicates that morphine was reliably established as a discriminative stimulus. Response rates for food were modestly impaired by the training dose of morphine (3.2 mg/kg, s.c. and 1.8 mg/kg, i.v.) compared to saline. Subcutaneous morphine produced dose-dependent responding on the drug-paired lever (ED$_{50}$ = 1.247 mg/kg, data not shown). Substitution curves were generated for morphine (Figure 6.1) and EM analogs (Figures 6.2-6.4) in tests where both levers delivered food. Table 6.2 shows relative ED$_{50}$’s after between-session and within-session cumulative injections of morphine or EM analogs. Cumulative injections produced slightly more potent ED$_{50}$ values for morphine and analogs compared to injections made between sessions. EM analogs fully substituted for morphine under both injection paradigms, but only morphine ($F[5, 29]= 9.467, p< 0.0001$, Figure 6.1), and to a lesser extent analog 1 ($F[5, 26]= 3.216, p=0.0216$, Figure 6.2) produced response rate deficits compared to vehicle. Response rates for food were not impaired by analog 2 ($F[5, 28]= 1.391, p=0.2578$ n.s., Figure 6.3) or analog 4 ($F[5, 29]= 2.063, p=0.0991$ n.s., Figure 6.4), at doses that fully substituted for morphine.
Figure 6.1 Drug discrimination training in male rats. a. Male rats were trained to discriminate morphine (3.2 mg/kg, s.c.) from vehicle (s.c.) after approximately 20 sessions. Rats were catheterized for i.v. injections, allowed to recover, and continued training to discriminate morphine (1.8 mg/kg, i.v.) from vehicle (i.v.) injections. Half the rats were trained to press the right lever when pre-injected with morphine, and the left lever when pre-injected with vehicle, and vice versa for the other half. The dashed lines indicate the training criteria of 90% drug-appropriate responding. b. During test sessions in which both levers actively delivered food, rats dose-dependently responded on the drug-paired lever for morphine after bolus injections made between sessions, or cumulative injections made within a single session. c. Morphine modestly impaired reinforcement rates for food during training. d. During test sessions morphine disrupted responding, especially at higher doses. *, ***, p< 0.05, 0.001 compared to vehicle

Table 6.2 Discriminative stimulus effects of EM analogs in male rats trained to discriminate morphine injections from saline. Values represent the % maximum morphine-appropriate responding, ED50 (mg/kg, i.v. [SEM]) after bolus injections made between sessions, or cumulative injections made within a single session. Response rate impairment shown as a percent of vehicle responding and SEM of 5-6 rats/group.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Maximum % drug-appropriate responding</th>
<th>ED50</th>
<th>Maximum % drug-appropriate responding</th>
<th>ED50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>100</td>
<td>0.879(0.365)</td>
<td>100</td>
<td>0.701(0.046)</td>
</tr>
<tr>
<td>EM analog 1</td>
<td>97.2</td>
<td>2.320(0.328)</td>
<td>98.2</td>
<td>1.201(0.170)</td>
</tr>
<tr>
<td>EM analog 2</td>
<td>98.0</td>
<td>1.631(0.205)</td>
<td>100</td>
<td>0.830(0.131)</td>
</tr>
<tr>
<td>EM analog 4</td>
<td>94.9</td>
<td>2.104(0.283)</td>
<td>89.0</td>
<td>1.765(2.333)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Response rate impairment %</th>
<th>Response rate impairment SEM</th>
<th>Response rate impairment %</th>
<th>Response rate impairment SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>77.1</td>
<td>11.6</td>
<td>54.5</td>
<td>19.1</td>
</tr>
<tr>
<td>EM analog 1</td>
<td>30.4</td>
<td>11.4</td>
<td>-0.3</td>
<td>11.7</td>
</tr>
<tr>
<td>EM analog 2</td>
<td>21.1</td>
<td>8.8</td>
<td>30.6</td>
<td>27.0</td>
</tr>
<tr>
<td>EM analog 4</td>
<td>15.1</td>
<td>10.2</td>
<td>0.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Figure 6.2 Discriminative stimulus and response rate effects of EM analog 1 in morphine-trained rats. The top panel shows morphine-appropriate lever responding during test sessions in which analog 1 was administered with bolus injections made between sessions or cumulative injections made within a single session. The bottom panel shows response rates for food (pressings/min) were modestly impaired by between session injection of analog 1, but not after cumulative injections. *p< 0.05 compared to vehicle.

Figure 6.3 Discriminative stimulus and response rate effects of EM analog 2 in morphine-trained rats. The top panel shows morphine-appropriate lever responding during test sessions in which analog 2 was administered with bolus injections made between sessions or cumulative injections made within a single session. The bottom panel shows response rates for food (pressings/min) were not affected by analog 2 after either injection paradigm.
Persistent substitution and Naloxone-reversible effects of EM analogs. Co-administration of naloxone with morphine or EM analog 4 prevented drug-lever responding in a dose-dependent manner. Naloxone (1.0 mg/kg, i.v.) completely blocked the DD effects of morphine and the substitution effects of EM analog 4 (Figure 6.5). A time course for the DD effects of vehicle, morphine, or EM analog 4 was generated by testing male rats for 5 min at 30 or 60 min intervals after injection (Figure 6.6). EM analog 4 substituted for morphine immediately and persisted for at least 4 hours. Morphine impaired reinforcement rates for approximately 30 mins ($F[1, 1]= 729.0, p= 0.0236$) compared to vehicle responding, while reinforcement rates after analog 2 ($F[1, 1]= 1.249, p= 0.4647$ n.s.) or analog 4 ($F[1, 1]= 5.444, p= 0.2578$ n.s.) were not altered at any time point compared to controls.

**Figure 6.4 Discriminative stimulus and response rate effects of EM analog 4 in morphine-trained rats.** The top panel shows morphine-appropriate lever responding during test sessions in which analog 4 was administered with bolus injections made between sessions, or cumulative injections made within a single session. The bottom panel shows response rates for food (pressings/min) were not affected by analog 4 after either injection paradigm.
Figure 6.5 Naloxone dose-dependently blocked drug-lever responding in rats injected with morphine or EM analogs. Rats trained to discriminate injections of morphine from vehicle were tested for naloxone sensitivity. Prior to injection with naloxone, rats responded appropriately when injected with i.v. vehicle (e.g. no drug-lever responding), morphine or analog 4 (~100% drug-lever responding). Co-administration of naloxone (0 - 5 mg/kg) with morphine (5.6 mg/kg) or analogs (5.6 mg/kg) dose-dependently blocked drug-lever responding, suggesting opioid receptors are required for the discrimination of morphine and EM analogs.

Figure 6.6 Time course for the discrimination effects of morphine and EM analogs in male rats. Morphine-trained rats were injected with i.v. vehicle, morphine (5.6 mg/kg), analog 2 (3.2 mg/kg) or analog 4 (5.6 mg/kg) and given regular 5 min test sessions in which both levers delivered food. Doses for the analogs were chosen based on doses that fully discriminated for morphine. In the top panel, rats injected with morphine, analog 2 or 4 persistently responded on the drug-paired lever for at least 4 hours. The bottom panel shows morphine suppressed reinforcement rates for approximately 30 minutes, while analogs 2 and 4 did not impair response rates for food. *, ** p< 0.05, 0.01.
**Drug discrimination effects of EM analogs in female rats.** Finally, female rats were trained to discriminate morphine injections from vehicle in a similar manner as described above for the males (Figure 6.7). Since catheter patency problems limited DD testing of the female rats, time course and antagonism testing were not possible. EM analogs fully substituted for morphine in female rats and the analogs were discriminated in a slightly more potent manner by females compared to males. At a dose of 3.2 mg/kg, all analogs fully substituted for morphine in females and this dose did not produce response rate deficits. Higher doses of morphine, EM analogs 1 and 2, but not analog 4, produced response rate impairment in females, however there was a clear dissociation between the substitution effects (3.2 mg/kg) of the analogs and the production of response rate deficits (5.6 mg/kg).

**Figure 6.7** Drug discrimination training in female rats. a. Female rats were trained to discriminate morphine (3.2mg/kg, s.c.) from vehicle (s.c.) after approximately 27 sessions. Rats...
were catheterized for i.v. injections and continued training to discriminate morphine (1.8 mg/kg, i.v.) from vehicle (i.v.) injections. Half the rats were trained to press the right lever when pre-injected with morphine and the left lever when pre-injected with vehicle, and vice versa for the other half. The dashed lines indicate the training criteria of 90% drug-appropriate responding. b. During test sessions in which both levers actively delivered food, female rats dose-dependently responded on the drug-paired lever for morphine or EM analogs. c. Morphine modestly impaired response rates for food during training. d. During test sessions, morphine and analogs 1 and 2 impaired response rates for food at the highest dose (5.6 mg/kg) tested, while analog 4 did not. It is important to note that the 3.2 mg/kg dose of the analogs fully substituted for morphine in female rats and this dose did not disrupt response rates. This suggests that the analogs may have slightly higher potency in female rats compared to males.

DISCUSSION

This study is the first to demonstrate that EM analogs fully substitute for morphine with less behavioral disruption. Rats trained to discriminate morphine from vehicle infusions (Figure 6.1) dose-dependently pressed the morphine-paired lever when pre-injected with EM analogs (Figure 6.2-6.4). The novel finding that EM analogs 2 (Figure 6.3) and 4 (Figure 6.4) did not impair response rates for food, at doses that fully substituted for morphine, suggests potential for a major improvement in opioid addiction pharmacotherapy, especially considering the analogs were not self-administered (Chapter 3) and did not produce CPP or LS (Chapter 2). Naloxone dose-dependently blocked the discriminative stimulus effects of EM analogs indicating that opioid receptors modulate the substitution effects of the analogs (Figure 6.5). Next, a time course for the DD effects of the analogs was assessed using 5-min sessions. Rats pressed on the morphine-paired lever immediately upon i.v. delivery of EM analogs 2 and 4 and these effects persisted at least 3 hours without significant response rate impairment (Figure 6.6). Female rats trained to discriminate morphine from vehicle infusions (Figure 6.7) also showed that the analogs substituted for morphine at doses that did not produce response rate impairment.
While the highest dose of morphine (5.6 mg/kg) and analogs 1 and 2, but not 4, disrupted response rates, all analogs fully substituted at 3.2 mg/kg, a dose that did not produce response rate impairment. Therefore, in both males and females, EM analogs produced morphine-appropriate responding without impairing response rates, however the potency of the DD effects of the analogs was slightly higher in females.

The training dose of morphine (3.2 mg/kg, s.c. and 1.8 mg/kg, i.v.) was carefully considered in this study (Young et al., 1992). Morphine at 3.2 mg/kg is commonly employed as training dose in DD protocols using s.c. injections, however there are no reports using i.v. injections in rats. This is likely due to the difficulty of maintaining catheter patency for the months required for DD training. We chose to continue with an i.v. training dose of morphine because the antinociceptive dose-response curves of EM analogs are similar to morphine only when injected i.v. One study comparing antinociceptive effects of s.c. vs. i.v. morphine showed that i.v. doses are more potent than s.c. (South et al., 2009), so the 1.8 mg/kg i.v. dose of morphine was chosen to avoid unnecessary disruption during DD training and to maintain sensitivity to the morphine cue. DD tests showed that the dose-response curve for i.v. morphine was slightly steeper than the s.c. morphine curve (data not shown) confirming minor route of injection differences.

There are several examples of opioids that substitute for morphine in the DD model. These include heroin, buprenorphine, fentanyl, oxycodone, methadone and methadone’s active metabolites (Beardsley et al., 2004; Craft et al., 1999; Vann et al., 2009; Young et al., 1992). Fentanyl and oxycodone are used frequently for pain management, while buprenorphine and methadone are often used for heroin addiction
maintenance therapy. A common effect of these opioids is that they impair response-rates for food in the DD model, promote reward behaviors (Beardsley et al, 2004; Martin et al, 2007; Mucha et al, 1982; Zhang et al, 2009), induce respiratory depression (Lewanowitsch, 2004), motor sensitization (Chapter 2), cognitive impairment (Hepner et al, 2002), and tolerance after chronic injections [Chapter 5 and see (Meltzer et al, 1978)]. While these compounds have genuinely beneficial effects for pain and addiction treatment, the dissociation of these key side effects could drastically improve treatment for opiate dependency.

There were nuanced differences between individual EM analogs and their substitution for morphine: all analogs fully substituted for morphine, but analogs 2 (Figure 6.3) and 4 (Figure 6.4) did not produce disruptive effects, while analog 1 (Figure 6.2) modestly decreased response rates and morphine abolished responding at high doses. This suggests that analogs 2 and 4, in particular, have favorable profiles for opioid addiction pharmacotherapy since they substituted for morphine without significant disruption. Since EM analogs substituted for morphine with less response-rate impairment, this can be viewed as an improved substitution / disruption ratio compared to other opioids that substitute for morphine. Indeed, the vast majority of compounds that substitute for morphine produce disruption at the same dose. For example, methadone and buprenorphine impaired response rates 50% and 65%, respectively, at doses that substituted for morphine in this model (Craft et al, 1999; Young et al, 1992). By comparison, EM analogs 1, 2, and 4 impaired response rates only 30%, 21%, and 15% after bolus injections, and 12%, 27%, and 3% after cumulative injections, respectively (Table 6.2). These improved substitution ratios argue that EM analogs could meet the
rigid requirement of substituting for morphine with less sedation, disruption, or rewarding effects such as self-administration.

In conclusion, the substitution effects of EM analogs for morphine were rapid, persistent for at least 4 hours, and were naloxone-reversible. EM analogs fully substituted for morphine in males and females and did not produce response rate disruptions at doses that fully substituted for morphine in either sex. The DD effects of EM analogs further confirmed the BBB findings from chapter 4 because only drugs that penetrate the BBB substitute for morphine in this model. Overall, the lack of CPP, LS (Chapter 3), and absence of SA escalation (Chapter 4) by EM analogs indicate low abuse liability. Combined with their persistent substitution for morphine in the DD model, EM analogs 2 and 4 could effectively treat opioid abuse disorders with reduced disruption.
Chapter 7: General Discussion

These studies focused on three pharmacodynamic characteristics of EM analogs:

1. The relative abuse liability of EM analogs compared to morphine; 2. The BBB penetration of the EM analogs; and 3. The mechanisms for the reduced side effects displayed by the analogs. Several findings support the argument that the analogs have low abuse liability despite BBB penetration (Chapter 4). In three distinct abuse liability models the analogs did not show reinforcing effects. Rats did not self-administer the analogs even under highly sensitive long-term access conditions (Chapter 3), the analogs did not induce conditioned place preference (CPP), and did not produce locomotor sensitization (LS) effects (Chapter 2). The analogs did not alter dopamine (DA) neuron morphology after place conditioning, while morphine shrank the size of DA neurons in the same model. The lack of DA adaptions induced by the analogs reflects their reduced reward effects. Since the analogs did not activate glial cells in the tolerance study (Chapter 5), a lack of immune reactivity could account for the reduced tolerance induced by the analogs. Morphine-substitution effects of EM analogs found in drug discrimination (DD) studies combined with the relative absence of reinforcing effects indicate promising potential for the treatment of opioid dependency (Chapter 6). Long-lasting μ-ORs agonist effects, oral availability, and low abuse liability strongly support use of the analogs for opioid maintenance therapy in addition to a variety of pain conditions.
Evidence that the EM analogs have reduced abuse liability (Chapters 2 and 3) support the case that the analogs could provide superior therapy for opioid dependency, with less abuse liability. The antinociceptive effects of EM analogs largely mirror their DD substitution effects (chapter 6), so it is reasonable to assume that a centrally mediated cue prompted by the antinociceptive, rather than rewarding effects of EM analogs accounted for their DD substitution effects (Figure 7.1). While rats responded on the morphine-paired lever when pre-injected with EM analogs, self-administration (SA) data suggests the analogs lack rewarding effects. Indeed, the available doses of EM analog 4 (1 and 3 mg/kg/infusion) during SA sessions corresponded roughly with the ED$_{20}$ and ED$_{80}$ found during DD test sessions (Figure 7.1). This indicates that rats experienced with morphine could discriminate EM analog 4, but analog 4 did not produce self-administrations at antinociceptive and discriminated doses under long-term access. Overall, the reduced abuse liability of EM analogs 4 is consistent with the reduced disruptive effects observed during morphine-substitution testing. These data suggest analog 4, and to a lesser extent, analog 2 could provide superior opioid substitution effects with less rewarding or disruptive effects. The analogs tested here could further understanding of a central issue for opioid maintenance therapy: how to maintain morphine-substitution without producing reward.
Figure 7.1  Comparison of the pharmacodynamic effects of morphine and EM analog 4. The left graph compares morphine antinociception and drug discrimination (DD) dose-response curves (left y-axis) to self-administration intake /h (mg/kg, right y-axis) during long-access SA sessions requiring high FR responding (FR3-5). Note that the hourly intake of morphine of 3.5 mg/kg/h at the 1 mg/kg/infusion dose is 2.8 times higher than the antinociceptive ED$_{50}$, 4 times higher than the ED$_{50}$ for DD, and greater than doses required for >90% responding on both measures. Thus, rats SA a larger amount of morphine than maximal doses producing antinociception or DD. The right graph compares the antinociception and drug discrimination dose-response curves (left y-axis) of EM analog 4 to self-administration intake /h (mg/kg, right y-axis) during long-access SA sessions. The hourly intake of analog 4 peaked at 1.1 mg/kg/h at the 3 mg/kg/infusion dose, but this was 1.4 times lower than the antinociceptive ED$_{50}$, and 1.9 times lower than the ED$_{50}$ for DD. The SA infusion doses of 1 and 3 mg/kg/infusion of analog 4 corresponded roughly with the ED$_{20}$ and ED$_{50}$ in the DD model, respectively. Similarly, the 1 mg/kg/infusion dose corresponded to the antinociceptive ED$_{20}$, and the 3 mg/kg/infusion dose produced maximal (100% MPE) antinociception. This suggests that even with long-term access to antinociceptive and morphine-discriminating doses of analog 4, the hourly SA intake remained below the level producing antinociception and morphine-substitution effects.

Three lines of evidence confirm that the analogs penetrate the BBB to produce their effects at centrally located sites. 1) The antinociceptive effects of peripherally administered EM analogs was blunted when animals were pretreated with intracerebroventricular (i.c.v.) Nlx-M, an opioid antagonist that does not cross the BBB. 2) Antinociceptive effects of EM analog 4 on the 52.5°C HP test indicated that centrally mediated pain behaviors such as licking or shaking the paws were inhibited by analog 4. 3) BBB penetration was confirmed by drug discrimination (DD) effects since animals
pressed the morphine-associated lever during test sessions when pre-injected with the analogs. This indicated that rats used a centrally located cue to prompt morphine-lever responding when injected with EM analogs. Central Nlx-M antagonism, HP activity, and DD effects together support central penetration of the analogs after peripheral administration. It is concluded that the inactivity in tests of reward are not due to lack of BBB penetration.

The reduced side effects of the analogs could be explained by differential glial cell reactivity and effects on dopamine neurons. Tolerance experiments performed here show that morphine induced robust increases in glial cell activation markers for microglia (Iba1, OX-42, and pp38) and astrocytes (GFAP). While the exact signaling mechanism for how glial cells exacerbate tolerance to morphine is still under examination, it is clear that glial cell inhibition attenuates tolerance to morphine. Purinergic (P2X) signaling may also contribute by glia-neuron crosstalk since P2X7 receptors were upregulated in spinal microglial cells from rats given chronic morphine, but not analogs. The microglial p38 kinase appears to be a common downstream target for all three proposed mechanisms that are activated by morphine: TLR, CGRP, and P2X7 signaling pathways. For example, microglial TLR4 activation by morphine activates p38 kinase to upregulate IL-1β mRNA levels (Hutchinson et al, 2010b). ATP-stimulated P2X7 receptors converge on the p38 pathway to increase production of IL-1β and IL-18 (Chen et al, 2012). CGRP activates this kinase through neuron to glia signaling (Wang et al, 2010). Since the analogs did not alter phosphorylated p38 levels in the spinal cord after chronic infusions, they likely avoided a host of proinflammatory signaling molecules known to counteract opioid antinociception. The lack of tolerance displayed by the analogs is consistent with this
evidence. Since pronociceptive glial signaling through p38 kinase activation has been linked to reward behaviors (Zhang et al, 2012), the results from the tolerance experiment support evidence for the lack of reinforcing effects produced by the analogs.

Signaling pathways responsible for reward may help clarify why the analogs have low abuse potential. Research spanning nearly 20 years shows chronic morphine or heroin injections can produce morphological, molecular, and physiological changes to dopamine (DA) neurons. DA neurons located in the VTA are disinhibited by acute injection of morphine which acts on inhibitory GABA neurons that normally exert tonic inhibition of DA neurons. Disinhibition of DA neurons increases DA release in limbic regions such as the nucleus accumbens (NAc) to produce a rewarding effect. On the other hand, chronic opioid exposure has been shown to decrease the release of DA in the NAc. This is due to hyperexcitable DA firing that essentially “burns out” the release of DA. These observations have been closely linked to reward-tolerance models showing opioids produce less reward after animals receive chronic injections. Chronic opioids produce visible reductions in the size of DA neurons in the VTA, an effect found in post mortem human opioid users, rats that self-administer heroin, and mice chronically injected with morphine (Mazei-Robison et al, 2011). This DA neuron “shrinkage” phenomenon has been closely associated with reward-tolerance effects of opioids. Here, DA neurons in the VTA from rats chronically injected with morphine or analogs were stained with tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of DA. Using unbiased methods, we sampled 2 subregions of the VTA, the paranigral (PN) and parabrachial pigmented (PBP) area, and found morphine, but not analogs, reduced the volume and surface area of these neurons. These regions have been found to project axons to the NAc.
core and shell regions (Ikemoto, 2007) associated with reward. Since the analogs did not shrink the size of DA neurons, the physiological properties of these neurons likely remained unchanged compared to controls. The analogs did not produce rewarding effects in CPP or SA models, consistent with the idea that the analogs do not produced reinforcing behaviors and do not alter DA neuron morphology after chronic injections.

Morphine and several related compounds (oxycodone and hydrocodone) were identified more than 90 years ago yet today remain widely used as treatments for severe pain. While these compounds effectively alleviate pain, chronic injections promote a host of negative side effects such as tolerance and risk for addiction. The prototype of this group, morphine, produces inflammatory glial cell signaling and physically reduces the size and function of DA neurons necessary for hedonic pleasure in response to natural rewards. By circumventing these adverse side effects, EM analogs show a highly favorable profile of potent, long lasting analgesia with reduced reward, tolerance, and associated neuronal and glial cell abnormalities. The use of the analogs for pain therapy is immediately clear, especially for neuropathic, inflammatory, and post-operative pain conditions that typically require multiple rounds of treatment. The stability and long duration of activity of the analogs suggest additional potential for chemotherapy-related pain, individuals that are positive for HIV/AIDS, and drug abusing populations with or without HIV/AIDS.

Successful opioid addiction maintenance therapy requires several pharmacodynamic effects displayed by the analogs. These include the capacity to substitute for morphine while showing reductions in: tolerance, proinflammatory effects, cognitive/motor impairment, motor deficits, respiratory depression, and reward. The long
duration of effects displayed by the EM analogs indicate strong potential for the
treatment of opioid dependency since fewer subsequent doses of analogs would be
required to maintain morphine or heroin substitution effects. Similarly, the lack of
tolerance and proinflammatory effects coupled with their oral availability suggests the
analogs could safely substitute for morphine in a clinically relevant manner. Methadone
maintenance therapy has been shown to effectively decrease mortality among opioid
addicts. However, these programs are often unavailable or have long waitlists (often 1-2
years), particularly in rural regions struggling with the opioid addiction epidemic of
recent years (Sigmon, 2014). Physicians are frequently unwilling to prescribe
buprenorphine for heroin addiction for fear of diversion to the illicit market. Novel
medications that do not produce adverse side effects, such as the analogs, could
significantly benefit this population. Indeed, a sustained release implantable formulation
of the analogs could greatly improve this field. Our studies show the analogs are highly
stable in saline (Zadina et al, in preparation) so an implantable capsule could theoretically
deliver opioid-substitution effects for many months. However, this technology awaits
further development and future clinical studies. In summary, the features of the analogs
strongly support use for analgesic and opioid maintenance therapy with low potential for
abuse.
BIBLIOGRAPHY


BIOGRAPHY

Mark Nilges lives with his wife Mary and daughter Lucy in New Orleans, LA. After receiving his Bachelor’s degree in psychology from Northern Illinois University in 2004, he worked as a Senior Research Associate for PsychoGenics, a biopharmaceutical research company based in Tarrytown, NY. Outside of the laboratory, Mark is an avid baseball enthusiast and a lifelong Chicago Cubs fan.