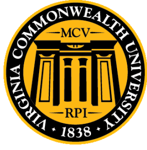

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Intrinsic Efficacy as a Determinant of Opioid Effectiveness in Treatment of Pain-Depressed Behavior

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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As I continue in my career, I hope to implement all that I have learned in the last five years. I'm so excited for the future!

Bones

Pepe

DeLuna

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List of Abbreviations

ACC……………………………………Articulate cingulate cortex

ANOVA……………………………………Analysis of variance

AP……………………………………Action potential

ASICs……………………………………Acid-sensing ion channels

ATP……………………………………adenosine triphosphate

BPI……………………………………Brief pain inventory

cAMP……………………………………Cyclic adenosine monophosphate

Ca2+……………………………………Calcium

CCI……………………………………Chronic constriction injury

CFA……………………………………Complete Freund's Adjuvant

CHO……………………………………Chinese hamster ovary cells

CL……………………………………Confidence limit

CNCP……………………………………Chronic non-cancer pain

CNS……………………………………Central nervous system

DAMGO……………………………………[D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin

DMSO……………………………………Dimethylsulfoxide

DOR……………………………………Delta-opioid receptor

DRG……………………………………Dorsal root ganglion

E……………………………………Efficacy

ED$_{50}$……………………………………Dose that produces 50% of the E$_{max}$

Emax……………………………………Maximum effect

EP50……………………………………Effective proportion

f …………………………………………………………………………Function transduction

FENT/NTX………………………………………………...….Fentanyl/naltrexone mixture

GABA.……..…………………..……..…………………..………γ-Aminobutyric acid

GI………..……..……………..……..……..……..……..……..………..Gastrointestinal

GIRK………………………G protein-coupled inwardly rectifying potassium channels

GPCR…………………………..…………………...…...G-protein coupled receptor

GTPγS……………………………………..Guanosine 5'-O-[gamma-thio]triphosphate

H^+……..…………………………..………..……………………………Hydrogen ions

HCl……………..…………..………..…………..………….…………..….Hydrochloride

IBS…..…………..……………..………..…….……...……….Irritable bowel syndrome

ICSS…..…………..…………..…………..……..…………..….Intracranial self-stimulation

ICR……..………..………..………..……….………..………Institute of cancer research

IC_{50}……..………..……..………..……..………..……..……..…..Inhibitory concentration

IMMPACT……………Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials

Interleukin-1β…………………………………………………….…………………….. IL-1β

Interleukin-6……………………………………………………….…………………….. IL-6

IP………..…………..……………..……………………………………….……Intraperitoneal

K_D…………………………………………………………………………...Drug affinity

K+……………………………………………………………………………. Potassium

KOR……………………………………………………………….…Kappa-opioid receptor

LA……………………………………………………………………………….…Lactic acid

[L] ……………………………………………………..…………Ligand concentration

MOR……………………………………………………………………. Mu-opioid receptor

MPI……………………………………………………Multidimensional pain inventory

MPQ………………………………………………………….McGill Pain Questionnaire

Na^{+}…………………………………………………………………………………Sodium

NIH…………………………………………………………National Institutes of Health

NMDR……………………………………………………N-methyl-D-aspartate receptor

NRS……………………………………………………………………Numerical rating scale

NSAID……………………………………………Non-steroidal anti-inflammatory drug

PAG………………………………………………………………………Periaqueductal grey

PAN……………………………………………………………………Primary afferent neuron

PB…………………………………………………………………………………Pain behavior

PDB…………………………………………………………………Pain-depressed behavior

PDI.. …………………………………………………………………Pain disability index

PDN………………………………………………………………Painful diabetic neuropathy

PFC……. ………………………………………………………………………Prefrontal cortex

PKA……………………………………………………………………………Protein kinase A

PGE2………………………………………………………………prostaglandin E2 receptor

PSB……………………………………………………………………Pain-stimulated behavior

PS……………………………………………………………………………………Pain stimulus

QOL…………………………………………………………………………………Quality of life

RA………………………………………………………………………Receptor activation

R_t……………………………………………………………Number of receptors in a system

ROS………………………………………………………………………Reactive oxygen species

RVM……..…………………………………………………..………Rostroventral medulla

SC………………………………………………………………..……Subcutaneous

SNI…………..………..…………..……….………….………….…………..…Spared nerve injury

SNL..…………..……………….………….…………..…….………..Spinal nerve ligation

SS-I…..……..……...……..……..…………..……..………..……..Somatosensory cortex

TM_3………………………………………………………….…....Transmembrane region 3

TM_7………………………………………………………….…....Transmembrane region 7

TNF-α……………………………………………………………Tumor necrosis factor α

TRVP……………………………………………Transient Receptor Potential Vanilloid 1

UR..…………………………....…………………………....….Unconditioned response

US..………………..……………..…………………..……………Unconditioned stimulus

VAS………..…………………………………………………………..Visual analog scale

VRS……………………………………..………………………Visual analog scale

List of Drugs

Clinically available single-molecule opioids (listed from highest to lowest MOR efficacy)

Methadone

Fentanyl

Morphine

Hydrocodone

Buprenorphine

Nalbuphine

Naltrexone

Fentanyl/naltrexone (FENT/NTX) mixtures (listed from highest to lowest MOR efficacy)

FENT/NTX 100:1

FENT/NTX 56:1

FENT/NTX 32:1

FENT/NTX 10:1

FENT/NTX 3.2:1

FENT/NTX 1:1

Novel single-molecule opioids (listed from highest to lowest MOR efficacy)

DC-01-128.1 (3-((1R,5S,9S)-2-Phenethyl-9-((Z)-prop-1-en-1-yl)-2-azabicyclo [3.3.1]nonan-5-yl)phenol)

DC-01-76.2 (3-((1R,5S,9S)-2-Phenethyl-9-((Z)-prop-1-en-1-yl)-2-azabicyclo [3.3.1]nonan-5-yl)phenol)

EWB-3-14 (1S,5S)-(-)-2, (1S,5S,9S)-(-)-8, and (1R,5S,9R)-(+)-15)

JL-02-0039 (3-((1S,5R,9R)-9-(2-Hydroxyethyl)-2-phenethyl-2azabicyclo[3.3.1]nonan-5-yl)phenol)

NAQ (17-Cyclopropylmethyl-3,14b- dihydroxy-4,5a-epoxy-6a-[(30 isoquinolyl)acetamido]-morphinan)

DC-01-76.1 (3-((1R,5S,9S)-2-Phenethyl-9-vinyl-2-azabicyclo [3.3.1]nonan-5-yl)phenol)

EG-1-203 (3-((1S,5R,9R)-2-phenethyl-9-propyl-2-azabicycloij3.3.1]nonan-5-yl)phenol)

EG-1-230 (3-((1S,5R,9R)-9-((E)-3-hydroxyprop-1-en-1-yl)-2phenethyl-2 azabicycloij3.3.1]nonan-5-yl)phenol)

Non-opioids

Ketoprofen – the nonsteroidal anti-inflammatory drug (NSAID) is a non-selective cyclooxygenase (COX_1 and COX_2) inhibitor

U69593 – centrally acting kappa opioid receptor agonist

Abstract

Intrinsic Efficacy as a Determinant of Opioid Effectiveness
in Treatment of Pain-Depressed Behavior

By: Edna J. Santos

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University (2023).

Advisor: S. Stevens Negus, PhD
Professor, Department of Pharmacology and Toxicology

Pain is a major public health concern that is commonly associated with behavioral depression, and a major goal in pain treatment is alleviation of pain-related behavioral depression. High-efficacy mu opioid receptor (MOR) agonists (e.g. fentanyl, morphine, oxycodone) are effective to treat pain, but their use is limited by side effects that not only endanger the patient but may also obscure analgesic rescue of pain-depressed behavior. The ongoing epidemic of opioid abuse and overdose deaths has stimulated research to discover non-opioid alternative analgesics; however, this search neglects the clinical potential of safer intermediate-efficacy MOR agonists (e.g. buprenorphine). The objective of the work presented in this dissertation was to test the hypothesis that intermediate- and low-efficacy MOR agonists would be more effective than high-efficacy MOR agonists to produce antinociception in assays of pain-depressed behavior at doses that do not produce motor disruption. To accomplish this, two main goals were achieved. First, we sought out to determine the effects of three classes of opioids to study MOR efficacy [listed from high- to low-efficacy] (1) clinically available single-molecule opioids [methadone, fentanyl, morphine, hydrocodone, buprenorphine, nalbuphine, naltrexone], (2) fentanyl/naltrexone (FENT/NTX) mixtures [100:1, 56:1, 32:1, 10:1, 3.2:1, 1:1], and (3) novel single-molecule opioids [DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, NAQ,

DC-01-76.1, EG-1-203, EG-1-230]. Second, we sought out to validate and establish two novel assays of pain-depressed behavior as a means of better preclinical-to-clinical translational outcomes (1) climbing, and (2) horizontal locomotor + vertical barrier behavior. The work accomplished in this dissertation can be split into three parts. First, in **Part I**, we studied MOR efficacy as a determinant of horizontal locomotor activity as a “pain-independent” behavior and results determined an efficacy-, dose-, and time-dependent opioid effect on locomotor activity in female and male ICR mice. Second, in **Part II**, we studied MOR efficacy as a determinant of antinociception in two assays of pain-depressed behavior by using the acute pain stimulus intraperitoneal (IP) lactic acid. Results determined that mouse climbing is a low efficacy requiring assay because it was too sensitive to mu-agonist induced effects to determine alleviation of the pain-depressed behavior; however, the locomotor + barrier assay was a high efficacy requiring assay because alleviation of the pain-depressed behavior was determined with intermediate- and low-efficacy opioids. In particular, a better window of opportunity to determine alleviation of the pain-depressed behavior at doses that did not alter behavior on their own was with opioids that had much lower efficacy than buprenorphine. Third, in **Part III**, we studied the expression of experimental chronic pain models (complete Freund’s Adjuvant (CFA), laparotomy, and spared nerve injury (SNI)) in the locomotor + barrier assay and results determined that each chronic pain model produced different magnitude and duration of behavioral depression. Overall, the work presented in this dissertation supports the hypothesis that (1) intermediate- to low-efficacy opioids provide the greatest window of opportunity to determine alleviation of the pain-depressed behavior, and (2) pain-depressed behaviors should be considered when studying novel analgesics.

Chapter one

Introduction

1.1. Opioid background/history

"The story of the opium poppy is almost as old as man" states author Sam Quinones in his famous 2015 book "Dreamland: The True Tale of America's Opioid Epidemic" (Quinones, 2016). This chapter will begin by introducing terminology that is important for understanding the history of opioids. First, *opium* refers to the fluid obtained from the poppy plant; second, *opiate* refers to a substance derived from opium; and third, *opioid* refers to a substance with opiate-like actions, but not derived directly from the poppy plant. These terms have become interchangeable; however, defining them is important to better understand their history. In section 1.1., each term will be used; however, later in this dissertation, all compounds will be referred to as <u>opioids</u>.

The use of opium for analgesic purposes has been a practice for centuries. The poppy plant, *Papaver somniferum,* nowadays found in Asia, the middle east, and Latin America, contains a fluid called opium, and more than 20 alkaloids such as morphine can be found in the opium that is excreted from the poppy plant. The earliest documented use of opium can be traced back to around 3,400 B.C. in lower Mesopotamia, and soon, demand for it increased as many countries began to grow and process opium themselves (Brownstein, 1993). This was because the powerful properties of opium were discovered such as pain relief, sleep induction, and stomach aid. Interestingly, the fact that opium was habit-forming, often called "poisonous," was known, but the positives outweighed the potential negatives. Fast forward to the early 1800s, when German pharmacist assistant Frederich Sertürner reported the isolation and crystallization of a pure substance from

opium and named it morphine for Morpheus, the Greek God of sleep and dreams (Brownstein, 1993). Since the isolation of morphine, its use in the medical field began to spread like wildfire, mainly for the treatment of pain (e.g., treating injured soldiers during the American Civil War). However, as time went on, morphine began to be used recreationally, and attempts to find a nonaddictive opioid with the therapeutic properties of morphine emerged. In 1874, English chemist C.R. Alder Wright was attempting to synthesize a nonaddictive version of morphine when he synthesized the opioid diacetylmorphine (Wright, 1874). While not much came from the discovery of C.R. Alder Wright, diacetylmorphine would soon be brought up to light again by German chemists Felix Hoffmann and Heinrich Dreser. In 1898, the chemists working for the German pharmaceutical company Bayer synthesized diacetylmorphine and named it heroin. They named it heroin based on the German word *heroisch,* which means "heroic," and this name was fitting, as heroin was marketed as a "wonder drug" for its supposed nonaddictive properties. Heroin, the new wonder drug, could be used for the treatment of pain, cough suppression, and as an antidiarrheal (Sneader, 1998). While this marketing practice seemed to work, it was soon discovered that heroin was addictive, and in fact, more potent than morphine (Brownstein, 1993).

Throughout the years, further attempts to synthesize opioids with little to no side effects has been of interest; however, most attempts have failed. Thus, a main goal of this dissertation work was to study a different class of opioids, low-efficacy opioids, which are hypothesized to retain the analgesic properties without many of the deleterious side effects. Sections 1.2-1.3 will discuss the diversity of opioid receptors and mechanism of action to better understand how opioids produce their effects. In sections 1.4-1.5, the use

of opioids for the treatment of pain and how this has played a role in preclinical animal research will be discussed further.

1.2. Opioid receptors, mechanism of action

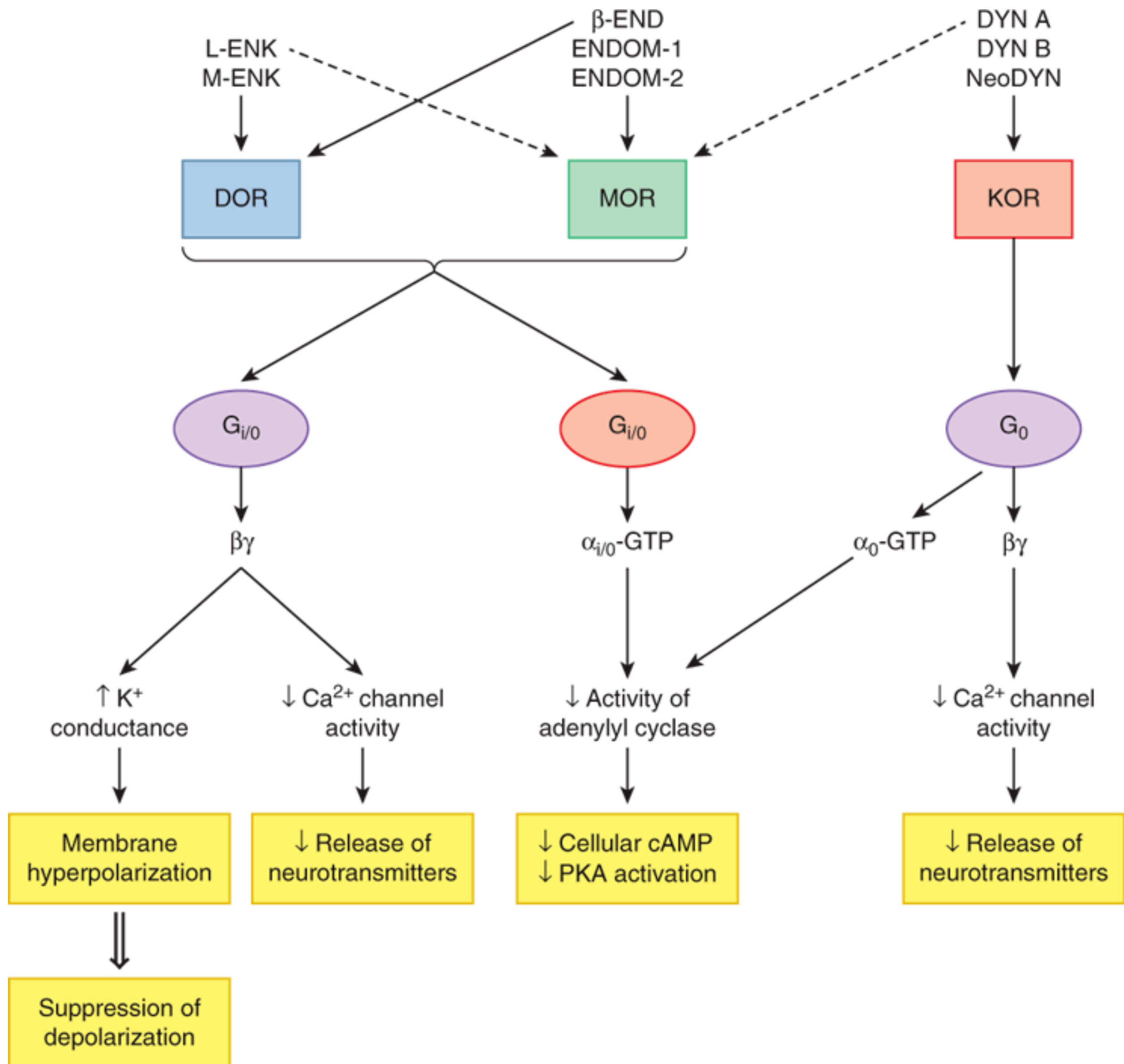
Three main types of opioid receptors have been identified, and they are the 1) mu-opioid receptor (MOR) 2) kappa-opioid receptor (KOR) and 3) delta-opioid receptor (DOR). Each has specific endogenous and exogenous ligands that bind their respective receptor, each produces different effects, and each is localized in different areas throughout the nervous system (Yaksh and Wallace, 2017). Studies helped identify these receptors through the identification of endogenous ligands that bind them (Gilbert and Martin, 1976; Hughes et al., 1975) them as seen in **Table 1.2.** This dissertation work was based on studying MOR ligand efficacy as a determinant of opioid effectiveness in treating pain. Thus, for simplicity, the focus of this chapter moving forward will be on MOR.

Table 1.2: Opioid receptors and ligand pathways.

Receptor	MOR	KOR	DOR
Endogenous ligand pathway	Pre-POMC ⬇ POMC ⬇ B-endorphin	Pre-dynorphin ⬇ Prodynorphin ⬇ Dynorphin A, Dynorphin B, and alpha-neoendorphin	Pre-proenkephalin ⬇ proenkephalin ⬇ Met-enkephalin and Leu-enkephalin

Mu-opioid ligands bind the MOR, which is a G-protein coupled receptor (GPCR). GPCR's are seven-transmembrane receptors that are embedded in the cell membrane. Some characteristic that all GPCR's share include an extracellular N-terminus and an intracellular C-terminus group. MOR's have a binding pocket between TM_3-TM_7 that allows for MOR selective ligands to bind the pocket. Specifically, MOR have three intra- and three extracellular loops, and ligand selectivity for MOR is attributed to extracellular loop 1 and 3 (Yaksh and Wallace, 2017). MOR GPCRs are coupled to the pertussis toxin-sensitive $G_{i/o}$ family of G-proteins. The G-protein complex has three regulatory subunits: alpha (α), beta (β), and gamma (γ). When a MOR agonist binds the receptor, a series of signaling cascades is activated. First, it recruits the $G_{i/o}$, α, β, γ complex to the GPCR. Among further activation, the complex is divided into two main components. The first pathway is the β-γ complex, and this is responsible for 1) activating G protein-coupled inwardly rectifying potassium K+ (GIRK) channels, which causes membrane hyperpolarization and inhibits tonic neuronal activity, 2) it inhibits Ca2+ channels from opening, thus causing an overall decrease in neurotransmitter release from the synaptic vesicles. The second pathway is the $\alpha_{\text{-}i/o}$ complex, and this is responsible for inhibiting adenylyl cyclase, thereby reducing conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and downstream cAMP-induced Protein kinase A (PKA) activity, among other effects. This schematic is represented in **Figure 1.2** (Yaksh and Wallace, 2017) which is taken from Goodman and Gilman's The Pharmacological Basis of Therapeutics 13th edition.

Figure 1.2 Receptor specificity of endogenous opioids and effects of receptor activation on neurons.



Source: Laurence L. Brunton, Randa Hilal-Dandan, Björn C. Knollmann: Goodman & Gilman's: The Pharmacological Basis of Therapeutics, Thirteenth Edition: Copyright © McGraw-Hill Education. All rights reserved.

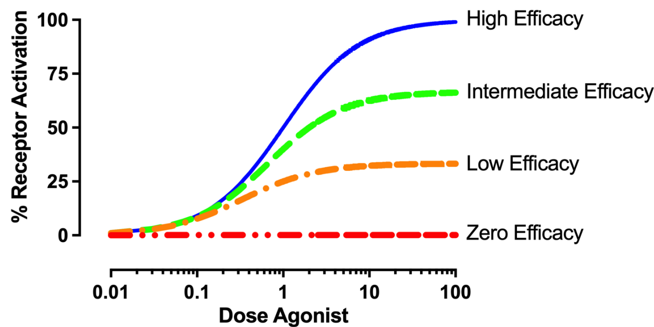
1.3. Opioid pharmacodynamics

When a ligand binds a receptor, two pharmacodynamic processes occur, and they are 1) affinity and 2) efficacy. Affinity refers to how well a drug molecule binds the binding site of a receptor, and efficacy refers to the capacity of a drug molecule to activate a receptor and produce an effect (Blumenthal, 2017). It is important to note that a drug molecule can have both affinity and efficacy for a receptor, but not all drugs have efficacy to produce an effect. Affinity and efficacy are variables that are part of the receptor occupancy theory, which explains overall drug effects on a system as seen in **Equation 1.3.** where RA is the total level of receptor activation in a population of receptors, f is the transduction function that describes the efficiency with which drug activates signaling at each receptor, R_t is the number of receptors in the system, E is the efficacy of the drug at the receptor, [L] is the ligand concentration or drug dose, and K_D is the affinity of the drug for the receptor. While all variables are important to consider, the work completed in this dissertation was mainly focused on efficacy, however, some focus was also placed on receptor selectivity and the reasons behind this will be described next.

Equation 1.3: Receptor occupancy theory

$$RA = f\left[R_t \times E \times \left(\frac{[L]}{[L] + K_D}\right)\right]$$

In relation to efficacy, a key determinant of the effects produced by any given MOR ligand is the relationship between two factors: (a) drug efficacy to activate MOR-coupled signaling mechanisms, and (b) efficacy requirements for different MOR-mediated effects (Selley et al., 2021). This relationship is illustrated in **Fig. 1.3.1.**



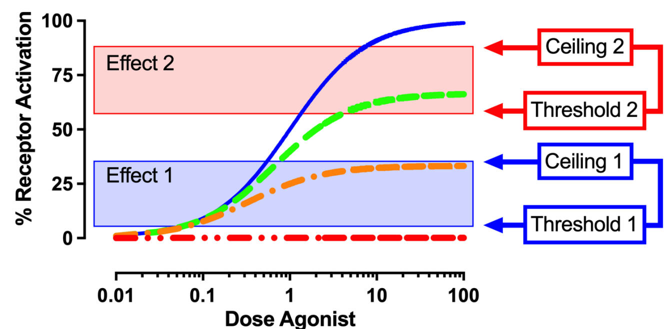


Figure 1.3.1: Dose and efficacy of the drug and efficacy requirement of the effect as determinants of drug effects. Drugs produce their effects by acting on a population of target receptors in the biologic system to which they are administered. For drugs with both affinity and efficacy at the target receptor, receptor theory predicts that increasing drug doses will produce increasing levels of receptor occupancy and activation. (A) Peak levels of total receptor activation are determined by drug efficacy, such that drugs with higher efficacy will produce higher plateau levels of receptor activation than drugs with lower efficacy. Abscissa: Drug dose in arbitrary units. Ordinate: % Total Possible Activation of all available receptors. (B) Different effects mediated by the receptor require different levels of receptor activation for their expression. Effects with a low efficacy requirement (Effect 1) require low levels of receptor activation to surpass the threshold and reach the ceiling for their expression. Even low-efficacy drugs have sufficient efficacy to produce these effects. Effects with a high efficacy requirement (Effect 2) require higher levels of receptor activation to surpass the threshold and reach the ceiling for their expression. High-efficacy drugs can produce sufficient receptor activation to produce these effects, although this will require higher doses than for effects with low efficacy requirements. Lower efficacy drugs may have sufficient efficacy to surpass the threshold but not reach the ceiling and may therefore function as partial agonists for these effects. Even lower efficacy drugs may lack sufficient efficacy even to reach the threshold, in which case they will function as antagonists.

For any in vitro or in vivo test system, increasing drug doses will produce increasing MOR occupation and increasing levels of MOR activation. Maximal receptor activation plateaus at high doses that saturate receptors, and high-efficacy MOR ligands will produce higher plateaus than lower efficacy ligands. Within this framework of dose- and efficacy-dependent MOR activation, different effects require different thresholds of receptor activation for their expression and are constrained by different ceilings imposed by either experimental or biologic limits. Together, the threshold and ceiling for a given effect define its efficacy requirement, which specifies the range of receptor-activation levels across which increasing doses will produce increasing effect. Moreover, the degree to which different effects have different efficacy requirements can be exploited in drug development, because low efficacy drugs may have sufficient efficacy to produce some therapeutic effects with low efficacy requirements but lack sufficient efficacy to produce some undesirable effects with high efficacy requirements.

In relation to receptor affinity (K_D), this refers to how well a drug molecule is able to bind (how “tight” a drug molecule binds) the receptor, thus, a drug molecule with high affinity will have a low K_D value and will bind a greater number of receptors at a lower concentration than a drug molecule that has a low affinity and high K_D value. Therefore, drugs that are selective for a particular receptor of interest will bind with high affinity to that receptor of interest vs other types. Of interest to the work presented in this dissertation was placed on studying low-efficacy opioids as potential useful analgesics. It is important to note that there are two clinically available low-efficacy opioids (e.g. buprenorphine, nalbuphine) (Fishman and Kim, 2018; Khanna and Pillarisetti, 2015;

Narver, 2015; Zeng et al., 2015); however, their poor MOR selectivity is a constraint for their use and that presents a problem because analgesia is a MOR mediated effect.

Thus, to better evaluate the role that MOR ligand efficacy and selectivity plays in the studies completed, three subcategories of opioids were examined throughout this dissertation work, with a specific focus on intermediate- and low-efficacy opioids. The subcategories of opioids were as follows 1) seven clinically available single-molecule MOR ligands, 2) a series of drug mixtures composed of the high-efficacy MOR agonist fentanyl and antagonist naltrexone, and 3) eight novel MOR selective, low-efficacy single-molecule opioids. Each will be discussed in further detail below, and data shown in tables represent data for maximal stimulation of [^{35}S]GTPɣS binding for all compounds in mouse-MOR expressing Chinese hamster ovary (CHO) cells (hydrocodone* is the exception because it was tested in CHO cells that expressed human-MORs) as an in vitro measure of relative efficacy.

1.3.1. Clinically available single-molecule opioids

The clinically available single-molecule opioids that were studied were six agonists and one antagonist. Importantly, the agonists studied have been used for the treatment of pain, and the role of MOR agonists in pain management will be further discussed in **section 1.7.** The agonists, or drug molecules that activate a receptor, were chosen because of their relative efficacy differences and clinical use and they were (from high- to low-efficacy): methadone, fentanyl, morphine, hydrocodone, buprenorphine, and nalbuphine. The antagonist, or drug molecule that occupies the receptor but does not activate it, was chosen because of its very low efficacy for MOR and that was naltrexone. Their relative efficacy differences were previously measured by Dr. Dana Selley and

colleagues in an assay of ligand-stimulated [^{35}S]GTPγS binding in MOR-expressing CHO cells (Selley et al., 1998; Thompson et al., 2004). The Emax of each compound is expressed as a percentage of the maximum effect produced by the selective, high-efficacy MOR agonist DAMGO, and results are shown in **Table 1.3.1**.

Table 1.3.1: Single molecule opioids

Opioids	Emax (% DAMGO Emax)
Methadone	108 ± 4.1
Fentanyl	110 ± 1.1
Morphine	106 ± 3.1
Hydrocodone*	54 ± 6
Buprenorphine	43 ± 3.5
Nalbuphine	26.4 ± 1.6
Naltrexone	5.9 ± 0.7

1.3.2. Agonist/antagonist opioid mixtures

Our lab has developed a novel and highly flexible strategy for selective and precise control of net efficacy to activate MOR using agonist/antagonist mixtures (Cornelissen et al., 2018; Santos et al., 2022; Schwienteck et al., 2019; Selley et al., 2021). These mixtures are composed of a fixed proportion of fentanyl (high-efficacy MOR agonist) and naltrexone (MOR antagonist) (FENT/NTX). The proportion of fentanyl can then be varied to vary the net efficacy of the mixture (e.g. higher fentanyl proportions yield higher net efficacy). A series of FENT/NTX mixtures was studied to vary the fentanyl proportion and generate mixtures with high- to low-efficacy: 100:1, 56:1, 32:1, 10:1, 3.2:1, and 1:1. The relative efficacies of these mixtures were interpolated from data collected by Dr. Dana Selley with a range of FENT/NTX mixtures in the assay of ligand-stimulated [^{35}S]GTPγS binding (Selley et al., 2021) in MOR CHO cells, and results are shown in **Table 1.3.2**.

Table 1.3.2: Agonist/antagonist mixtures

Fentanyl/naltrexone mixtures	Emax (% DAMGO Emax)
100:1	64.3
56:1	51.2
32:1	38.6
10:1	17.6
3.2:1	6.9
1:1	N/D

1.3.3. Novel low-efficacy single-molecule opioids

As noted above, the poor selectivity of existing low-efficacy opioids is one constraint on their use. Via a collaboration with chemists at Virginia Commonwealth University (NAQ, synthesized by Dr. Yan Zhang and colleagues) and the National Institute on Drug Abuse Intramural Research Program (all other compounds, synthesized by Drs. Kenner Rice and Arthur Jacobson), a series of low-efficacy MOR-selective compounds were synthesized that aimed at targeting both efficacy and MOR selectivity. In receptor binding studies conducted by Dr. Dana Selley, all compounds had higher MOR selectivity than nalbuphine, with MOR selectivity >10-fold vs KOR and MOR selectivity >80-fold vs DOR (personal communication, Dana Selley). Additionally, the compounds displayed a range of relatively low efficacies. Specifically, the compounds studied were (from high- to low-efficacy): DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, NAQ, DC-01-76.1, EG-1-203, and EG-1-230 (Chambers et al., 2022; Gutman et al., 2020, Lutz 2023; *In Press*, Tom Prinsenzano personal communication). Their relative efficacy differences were again measured by Dr. Dana Selley in the assay of agonist-stimulated [^{35}S]GTPγS binding and are shown in **Table 1.3.3.** More information on these compounds will be discussed in Chapter 3.

Table 1.3.3: Novel low-efficacy opioids

Compounds	Efficacy (% DAMGO Emax)
DC-01-128.1	75.4 ± 3.8
DC-01-76.2	29.1 ± 0.8
EWB-3-14	20.8 ± 1.7
JL-02-0039	13.0 ± 1.5
NAQ	12.0 ± 1.4
DC-01-76.1	10.5 ± 0.8
EG-1-203	4.8 ± 0.6
EG-1-230	0.7 ± 0.8

1.4. Clinical pain assessment

Pain is a complex experience and a costly problem in the United States (Institute of Medicine, 2011). Pain is currently defined as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Raja et al., 2020). Pain can be categorized as (1) acute or (2) chronic and this can be the result of injury, surgery, illness, or disease. Acute pain is usually short lasting, sharp in sensation, lasts up to four-weeks, and usually resolves when the underlying condition is treated (IASP, web). Chronic pain is defined as lasting more than 3 months, is more complex to treat than acute pain, and a recent study suggests that 1 in 5 adults in the US suffers from chronic pain (Yong et al., 2022). That is because chronic pain can be reoccurring, can stop and begin at any time, and sometimes, causes are unknown thus making it difficult to treat. Untreated pain can affect overall quality of life, sleep, health, mood, and brain function. A main concern that is often associated with chronic pain is functional impairment, and a recent study suggests that 10% of US adults suffer from high-impact chronic pain that leads to work limitations (Yong et al., 2022). Functional impairment can be defined as an overall decrease in behavior, and examples include

missing work, reduced ability to take care of loved ones and/or self, reduced recreational activities (e.g. exercise, walking), and sleep disruption (Turk et al., 2016, 2003). Thus, a goal of treating and managing chronic pain is to be able to restore this functional impairment to allow for behaviors to return to normal.

In the clinic, pain intensity is measured by three main types of subjective reports. These include the use of the verbal rating scale (VRS), numerical rating scale (NRS), and visual analog scale (VAS). For example, the VAS is a blank 10-cm line with two end points that are labeled “no pain” to “worst pain imaginable”, and the patient is required to place a mark on the line suggestive of the pain they currently feel or felt the week before (i.e. depends on the question being asked). What is being measured is a numerical index, which is measured as the distance (in cm) from the lowest endpoint to where they placed their mark. There could also be other integrations to the VAS such as (1) a rating system from 0-10 (NRS), and (2) faces that may represent a current pain state based on the question being asked (VAS). While these subjective pain reports have been used for their ease, there are two main disadvantages. First, the subjective scales require the verbal capacity and understanding of the question, and while most people may have the capacity to understand and answer, this presents an issue with non-verbal patients (e.g. pediatrics, geriatrics). Second, these scales are one-dimensional, meaning that most of the questions being asked to relate to pain intensity, which *is* a component of the pain experience, but it is not all encompassing and leaves out important qualities such as the sensory and affective components of pain. To better get at this, the McGill Pain Questionnaire (MPQ) was developed, with the intension to encompass more components of the pain experience such as: sensory, affective, evaluative, and miscellaneous. While

the MPQ encompasses more components of the pain experience, one important aspect of the result of pain is forgotten: and that is the effect of pain on overall quality of life.

Clinically relevant pain is often associated with impaired function and behavioral depression, and a common goal of pain treatment is to alleviate these manifestations of pain and restore normal behavior (Cleeland and Ryan, 1994; Dworkin et al., 2005). While the use of electronic devices to monitor function (either in the clinic or outside the clinic) has become a useful tool over the years, its feasibility (e.g. for the researchers to get or the patients to obtain and/or use) hinders this as a potential outcome in every clinical pain trial (Haythornthwaite, 2013). Thus, functional impairment typically involves self-report measures to describe the extent to which pain interferes with daily activities, and this has been recommended by the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) as one of its six core domains for clinical trials (Dworkin et al., 2005). There are a few self-report questionnaires that are important and often recommended when considering overall physical functioning (Turk et al., 2016). First, the multidimensional pain inventory (MPI) is a category of 12 subscales that measures physical and psychosocial function and includes various aspects of everyday function such as life control, negative mood, and activity level (Dworkin et al., 2005). The "general activity scale" is a subscale of the MPI and has been reported to be more reflective of physical functioning because it considers a summary of activities performed outside of the home, work, social activities, and household chores (Haythornthwaite, 2013). Second, the Brief Pain Inventory (BPI) includes seven areas of interest such as general activity, mood, walking ability, enjoyment of life, and sleep (Cleeland and Ryan, 1994; Haythornthwaite, 2013). Third is the Pain Disability Index (PDI), which measures the

degree to which pain interferes with normal function (Haythornthwaite, 2013; Pollard, 1984). A main disadvantage to these questionnaires is that often, an integration of multiple questionnaires may be necessary to have an understanding of functional impairment in patients. For example, the BPI has an assessment of sleep activity and the MPI does not (Dworkin et al., 2005) thus both the MPI and BPI may be recommended, and this may prove to be burdensome to both the patient and clinician.

1.5. Preclinical pain assessment

There are two main key differences between pain assessment in the clinic vs in preclinical animal models, and they will be explained further. First, is the concept that in humans, the subjective experience of pain can be measured (as discussed in **section 1.4**.) because a human can verbally report that experience. However, in preclinical animal models, “pain” is not what is measured because animals are non-verbal, thus they cannot tell the investigator if they are feeling pain. Rather, what is being measured is a behavior as a consequence of a “noxious stimulus”, which is defined as a stimulus that damages or has the potential to damage normal tissue. Thus, nociception, is the neural process of encoding a noxious stimulus (Sandkühler, 2013). Second, acute pain is defined as short acting, and chronic pain is defined as having lasted ≥3 months. However, preclinical models of “pain” do not necessarily reflect these time points (Mogil, 2022). For instance, **Table 1.5.1.** shows the approximate duration of action of commonly used noxious stimuli (five of which will be discussed further in the next couple of sections).

Table 1.5.1: Duration of action of noxious stimuli

"Pain" stimulus	Duration of action	"Pain" duration category
von Frey filaments	Seconds	Acute
Hot water/plate	Seconds	Acute
IP acid	1-2 hours	Acute
Laparotomy	Hours to days	Chronic
Ipl CFA	Days to weeks	Chronic
SNI	Months to permanent	Chronic

Nociception in the presence of a noxious stimulus in preclinical animal models is observed using different categories of pain behaviors. The aims of this project focused on unconditioned behaviors, or unconditioned responses (URs), which can be defined as any stimulus-induced physiologic or behavioral responses that do not require learning for their expression (Negus, 2019). Unconditioned behaviors are elicited by unconditioned stimuli (US), and this relationship can be described by using the terms US→UR. In any assay of preclinical analgesic testing, two concepts are true. First, there is a "pain stimulus (PS)" that is delivered to the subject and defined as the independent variable. Second, the "pain behavior (PB)" is what is measured as a consequence of the PS and can be defined as the dependent variable. Unconditioned preclinical pain behaviors can be further subdivided into two additional categories. They are (1) pain-stimulated behaviors (PSB), which are defined as behaviors that increase in rate, frequency, or intensity in the presence of a noxious stimulus, and (2) pain-depressed behaviors (PDB), which are defined as behaviors that decrease in rate, frequency, or intensity in the presence of a noxious stimulus. In assays of PSBs, the noxious stimulus serves as the US to stimulate a pain behavior as the UR, and this can be diagrammed as PS➔PSB. An example is the warm-water tail-withdrawal assay used in rodents, in which water heated to temperatures

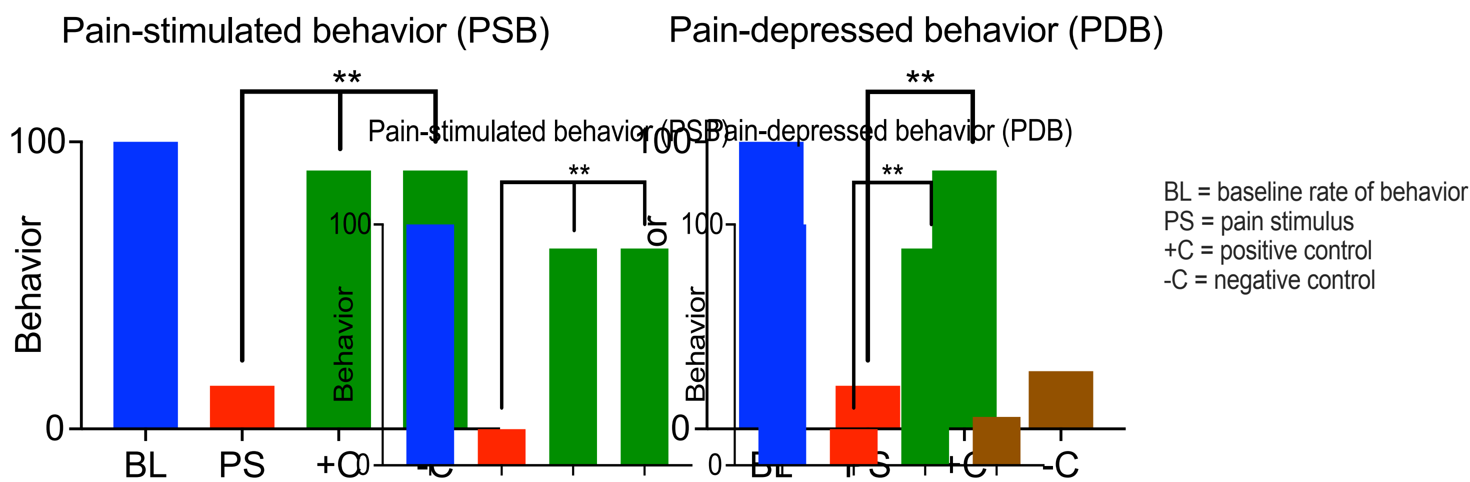
≥50°C serves as the PS to stimulate tail-withdrawal as the PSB. In assays of PDBs, the pain stimulus serves as a contextual stimulus to disrupt effectiveness of a non-pain US to stimulate a non-pain UR, and this can be diagrammed as PS↓[US➔UR]. As an example, the novel environment of an activity chamber can serve as a US that would normally elicit exploratory locomotor behavior by mice as the UR. Pretreatment of the subject with an intraperitoneal injection of dilute lactic or acetic acid (IP acid) as a noxious stimulus can serve as a contextual PS to disrupt this novelty-stimulated locomotion.

1.5.1: Pain-stimulated behaviors (PSB)

Pain-stimulated behaviors have been predominant in preclinical research for the discovery of novel analgesics (Mogil, 2022). In an assay of PSB, the PS is delivered, and the response is an UR, which are usually reflexive behavioral endpoints such as tail-withdrawal from heat or paw-withdrawal elicited by von Frey filaments to assess mechanical sensitivity. While there may be some utility for studying pain-stimulated behaviors, there are two main issues with them being predominant which have hindered the possibility of discovering novel tools and analgesics for the treatment of pain (Negus, 2019). First, while PSB measure reflexive behaviors and analgesics block or decrease these behaviors in preclinical models, analgesics are not used to block withdrawal reflexive behaviors in humans. For example, in humans, withdrawal reflexes are blocked during surgery; however, drugs used to block these withdrawal reflexes are not analgesics, but rather anesthetics. Thus, this shows that the behavior measured (withdrawal reflex) in preclinical animal models to test candidate analgesics does not translate to the measure tested in humans. Second, many non-analgesic drugs produce motor disruption (e.g. decrease behavior), and assays of pain-stimulated behavior are

more susceptible to false positive effects that are observed with candidate analgesics that produce motor impairing effects. A great example of this are the effects of the centrally acting kappa opioid receptor agonist (KOR) U69593. In an assay of PSB, U69593 looks like a candidate analgesic because it decreases the reflexive behavioral response measured (e.g. tail-withdrawal from noxious heat or abdominal stretching after administration of IP acid). However, centrally acting KOR agonists like U69593 are not effective as analgesics in humans (Lazenka, 2021); instead, their false-positive antinociceptive effects in preclinical studies are due to motor impairment (Negus et al., 2015) as observed in **Figure 1.5.** Thus, such drugs may show an analgesic-like decrease in pain-stimulated behaviors, but this decrease in behavior is due to motor depression and not analgesia. For these reasons, the focus of this dissertation work relied on studying pain-depressed behavior as a means of improving preclinical-to-clinical translational outcomes.

Figure 1.5: Analgesics effects produced in different preclinical behavioral assays.



1.5.2: Pain-depressed behaviors (PDB)

Preclinical research using experimental pain models can focus on parallel endpoints of pain-depressed behavior. For example, in a mouse climbing assay, the climbing chamber serves as the US to stimulate exploratory climbing behavior as the UR. If mice are pretreated with an injection of IP acid as a pain stimulus (the PS), then climbing is depressed (Santos et al., 2023). This IP acid-induced depression of climbing can serve as an example of a pain-depressed behavior, and drugs can then be evaluated for their effectiveness to restore pain-depressed behavior. There are two main advantages to studying pain-depressed behaviors as a category of endpoints for research on expression, mechanisms, and treatment of pain. First, preclinical-to-clinical translational research in any domain is optimized when preclinical studies measure endpoints homologous to clinically relevant human endpoints (González-Cano et al., 2020; Yu, 2011). The goal of potential analgesics is to restore or reverse selected behaviors that were decreased by a noxious stimulus (e.g. back to “normal” or baseline levels), and a main goal in humans patients suffering from pain is to restore behaviors that have been decreased due to ongoing pain states. Thus, as noted above, preclinical endpoints of pain-depressed behavior focus on behavioral depression, and this is homologous to clinical endpoints of pain-related functional impairment and behavioral depression in humans as noted in **section 1.4** (Cobos et al., 2012). Second, pain-depressed behaviors are not susceptible to false positive effects observed with drugs that cause motor impairment because effective analgesics will increase the expression of the pain-depressed behaviors, whereas drugs that produce motor impairment only exacerbate pain-related behavioral depression (Negus, 2019, 2013). Taking to account again the

example of U69593 in **section 1.5.1**., when tested alone, U69593 causes overall motor disruption that can result in false-positive effects in an assay of pain-stimulated behavior; however, in an assay of pain-depressed behavior, the target behavior is already decreased (and the goal is to restore that decrease in behavior), and U69593 fails to reverse this decrease in behavior and produce analgesia-like effects as observed in **Figure 1.5**. Because pain-depressed behaviors have been studied as potential endpoints that are more translationally relevant for studying candidate analgesics for the treatment of pain in preclinical animal models, different variables such as (1) pain stimulus/intensity (which can be considered the independent variable being manipulated) and (2) behavior being measured (which can be considered the dependent measure) are important factors to consider when studying potential pain-depressed behaviors.

1.5.3: Behavioral endpoints

Many different behaviors have been studied in mice and rats with the overall goal to determine reliable depression of behavior by different pain stimuli. For instance, in mice, different unconditioned behaviors have been decreased by increasing concentrations of IP acid as an acute pain stimulus. Behaviors studied in mice include climbing [measured as the amount spent climbing in a vertical chamber with scalable walls (Santos et al., 2023)], feeding [measured as amount consumed (Stevenson et al., 2006)], horizontal locomotion [measured as distance traveled or number of photobeam breaks (Stevenson et al., 2009)], voluntary wheel running [also measured as distance traveled (Cobos et al., 2012)], nesting behaviors [quantified using measures of the consolidation and shredding of nestlet material used to construct a nest (Diester et al., 2021; Garner et al., 2021; Negus et al., 2015)], burrowing [measured as amount of

substrate removed from a container over time (Makowska and Weary, 2016)], and cage-lid hanging [measured as the amount of time spent hanging from the wire lid of the home cage (Zhang et al., 2020)]. Unconditioned behaviors including feeding, locomotion, and burrowing have also been used in assays of pain-depressed behavior in rats (Craft, 2023; Kwilasz and Negus, 2012; Matson et al., 2007). Additionally, studies in rats have also used operant-conditioning procedures to assess pain-related depression of operant responding maintained by electrical brain stimulation, food delivery, and social access to another rat (Altarifi et al., 2015a; Baldwin et al., 2022). The selection of a behavioral endpoint for studies of pain-depressed behavior is influenced by several factors (Negus, 2013). Most importantly, the target behavior should be expressed at a high and stable level in the absence of pain, reliably depressed by experimental pain stimuli, and reliably restored by clinically effective positive-control analgesics but not by negative-control non-analgesics. Additionally, the precision and efficiency of the procedures is enhanced when the target behavior is an unconditioned behavior that does not require training and can be measured as a ratio variable amenable to parametric statistical analysis. This project included two novel behavioral endpoints that could serve as potential pain-depressed behaviors in mice, and they include (1) climbing in vertical chambers (**Chapter 4**) and (2) a combination of horizontal and vertical movement in the "locomotor + barrier" assay (**Chapters 5 and 6**). Each will be described in detail in subsequent chapters.

1.5.4. Pain States

1.5.4.1 Acute pain

By definition, acute pain states should be short in duration of action. In preclinical research, IP acid has been used as an acute visceral pain stimulus, and there are four

main reasons for its utility. First, IP acid models tissue acidosis, which is often observed in different pain states (Laura et al., 2008; Reeh and Steen, 1996). Second, IP acid serves as a strong pain stimulus with a duration of action of 1-2 hours, which is sufficient to observe depression of a wide variety of different behaviors (Bagdas et al., 2016; Baldwin et al., 2022; Diester et al., 2021; Negus et al., 2015; Patrick et al., 1999; Santos et al., 2023; Stevenson et al., 2009). Third, IP acid acts directly on primary nociceptors to activate the nociceptive pain pathway (Dawes et al., 2006; Ringkamp et al., 2013). Fourth, the decrease in behavior produced by IP acid can be selectively blocked by positive controls (e.g. ketoprofen), but not by negative controls (e.g. U69593) (Bagdas et al., 2016; Negus et al., 2015; Santos et al., 2023). For example, Negus et al (Negus et al., 2015) showed the effects of IP lactic acid as a pain stimulus on nesting behavior. They found that IP lactic acid required a 10-fold concentration to decrease nesting (0.032-0.32 mg/kg) because this effect was significant at 0.18% and 0.32%, with greater behavioral depression determined at 0.32% IP lactic acid, and that this effect lasted for 20-minutes. Accordingly, they also determined that the positive controls ketoprofen (an NSAID) and morphine (MOR agonist) were effective at blocking the IP acid-induced depression of behavior; however, the negative control U69593 (centrally acting KOR agonist) was not effective at blocking the IP acid-induced depression of behavior. The effectiveness of IP acid to elicit pain-related behavioral depression will be discussed in detail in **Chapters 4** and **5**.

1.5.4.2 Chronic pain

As mentioned in **section 1.4** of this Introduction, chronic pain is defined as pain lasting (or not resolved) for more than 3 months since its onset. In preclinical animal

models, different types of chronic pain manipulations are often studied because preclinical animal models provide a framework to be able to model different causes of pain in humans. For example, models such as complete Freund's Adjuvant (CFA), which is heat-killed mycobacterium that is injected into areas such as the paw, knee, or tail in a rodent, induce a localized (or systemic) inflammatory response (Berge, 2013) that can model inflammatory pain states such as arthritis. Models such as spared nerve injury (SNI), chronic constriction injury (CCI), or spinal nerve ligation (SNL) involve cutting or manipulating nerves which leads to peripheral neuropathy similar to that observed in humans due to injury, infection, or disease (Berge, 2013). These models have been effective to cause pain-depressed behavior. For example, intraplanar injection of CFA has been shown to decrease voluntary wheel running over a two-day period in mice (Cobos et al., 2012) and rats (Kandasamy et al., 2017). The laparotomy model of post-surgical pain has shown to decrease burrowing (Furumoto et al., 2021) and voluntary wheel running (Kendall et al., 2016). Cage-lid hanging behavior has been decreased by the chronic constriction injury (CCI) (Zhang et al., 2020) and by the spared nerve injury (SNI) (Pitzer et al., 2016) models of neuropathic pain in mice. These studies show the feasibility of studying different pain stimuli that model different pain etiologies to decrease or suppress a targeted behavior. The effect of CFA, laparotomy, and SNI models will be discussed further in **Chapter 6.**

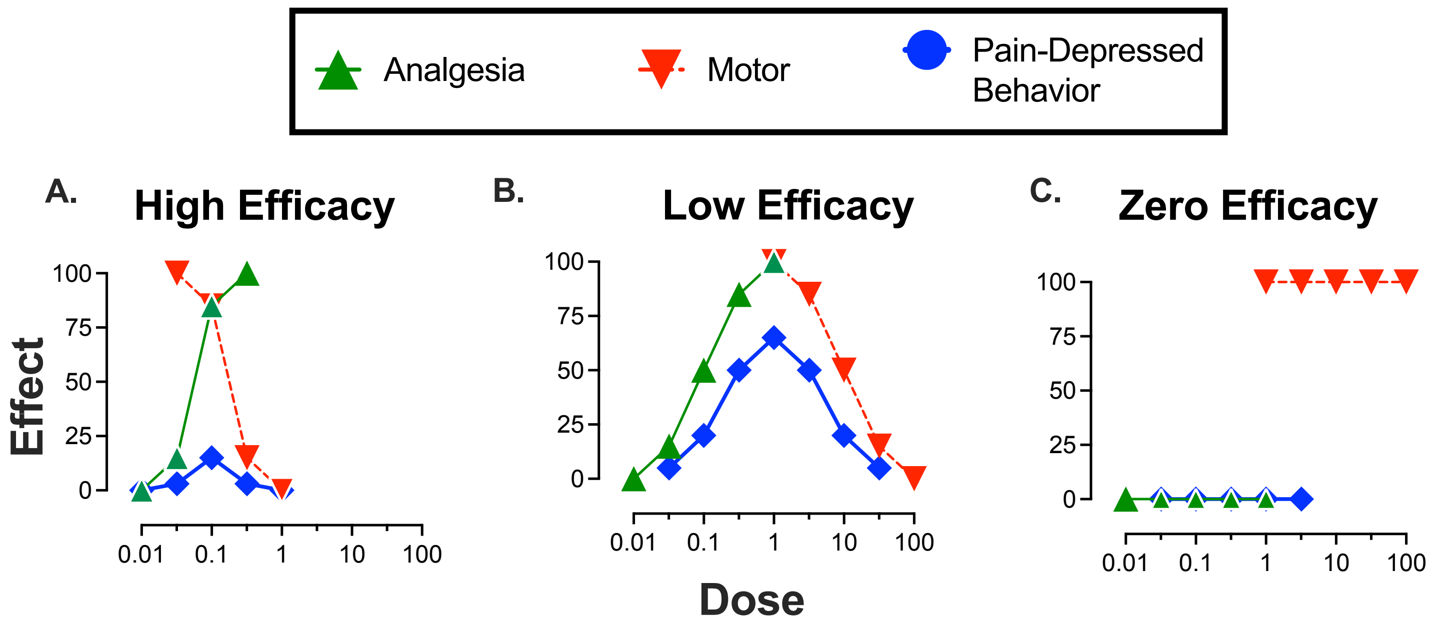
1.6. Opioid effects in models of pain-depressed behavior

Opioids are valuable analgesic drugs for treatment of a wide range of different pain states, but they are not uniformly effective to treat human pain, nor are they uniformly effective in animal models to alleviate pain-related behavioral depression. There are a

variety of reasons why opioids may or may not be effective to block pain-related behavioral depression, and opioid effectiveness can be influenced by variables that include the type of pain stimulus, intensity of the pain stimulus, or the behavior that is being measured. Opioid efficacy is another potential factor that could play a role in opioid effectiveness to block pain-related behavioral depression. A main reason why opioids may fail to restore pain-depressed behaviors may be due to motor disruption caused in the absence of the pain stimulus and this may be able to be explained by the following. In an assay of pain-depressed behavior, opioids are producing multiple effects that include both analgesia and motor disruption; however, in assays of pain-depressed behavior, analgesia and motor disruption tend to work in opposite directions, as seen in **Figure 1.6.** Specifically, opioids may produce both analgesic effects (which tend to increase expression of the pain-depressed behavior) and motor impairment (which tends to decrease expression of the target behavior), and opioid-induced relief of pain-related behavioral depression will reflect an integration of these two competing effects. For high-efficacy opioids (**Figure 1.6.A**), dose-effect curves are steep and potency to produce analgesia may be similar to or only slightly greater than potency to produce motor impairment. As a result, the potency window for production of analgesia without motor impairment is small, and the opioid may be relatively ineffective to alleviate pain-related behavioral depression. At the other extreme, opioid with zero efficacy (**Figure 1.6.C**) lack sufficient efficacy to produce either analgesia or motor impairment, and the absence of analgesic effects again result in poor effectiveness to alleviate pain-related behavioral depression. Between these extremes are intermediate- to low-efficacy opioids (**Figure 1.6.B**), which may have sufficient efficacy to produce both analgesia and motor

impairment, but with a larger potency difference between these two effects to permit a more robust restoration of pain-depressed behavior. For the work done in this project, the working hypothesis was that intermediate- and low-efficacy opioids would have sufficient efficacy to block pain-depressed behavior without causing motor disruption on their own. Because of this working hypothesis, the greatest window of opportunity to see relief of the pain-depressed behavior is hypothesized to be with low-efficacy opioids and data on this is discussed in **Chapters 4** and **5**.

Figure 1.6: Opioid effects in assays of antinociception



Opioid effects on behavior in the absence and presence of pain manipulations have been examined in a broad range of studies. For instance, it has been shown that nesting in mice can be disrupted by high doses of different opioids (e.g., morphine, oxycodone, buprenorphine) (Diester et al., 2021; Garner et al., 2021). In rats, operant

responding maintained by electrical brain stimulation (Altarifi et al., 2015a) and social reinforcement (Baldwin et al., 2022) is disrupted by 3.2 mg/kg morphine, and burrowing is disrupted by 3.0 mg/kg (Brust et al., 2016). The result of these studies suggest that motor disruption caused by opioids may hinder the ability to detect a restoration of pain-depressed behavior. However, in some instances, opioids can be effective to block pain-related behavioral depression at doses below those that cause motor disruption. In one study (Negus et al., 2015), for example, 0.32% IP acid was used as the pain stimulus and the behavior measured was nesting, and 1.0-3.2 mg/kg of morphine was effective to block the IP acid-induced depression of nesting. In this same study, it was determined that a different pain stimulus, unilateral injection of CFA, was also effective to decrease nesting, and 0.32-1.0 mg/kg morphine was effective to reverse this CFA effect at doses that did not alter behavior on their own. In another study (Garner et al., 2021), two different IP acid concentrations were used to produce different magnitudes of pain-depressed nesting in mice. When the lower concentration of 0.18% IP acid was used as the pain stimulus, 0.1-1.0 mg/kg morphine was effective to block the IP acid effects at doses that did not alter behavior when administered alone. However, when a higher acid concentration of 0.32% IP acid was used to produce a more severe depression of nesting, these low morphine doses were no longer effective to restore nesting, and higher doses that might have alleviated IP acid effects were sufficient to depress behavior on their own and obscure any analgesic restoration of pain-depressed behavior. In this case, the potency difference of morphine's effectiveness to block a pain-depressed behavior was dependent on the intensity of the noxious stimulus. It also has been shown that the laparotomy model of post-surgical pain was effective to decrease wheel running in mice, and buprenorphine

reversed this depression of behavior (Kendall et al., 2016). In rats, it has been shown that CFA can decrease behaviors such as burrowing and wheel running, and morphine was effective to reverse the effects at doses that did not cause motor disruption (Kandasamy et al., 2017; Rutten et al., 2014). Taken together, these results show that different variables such as opioid efficacy, pain stimulus intensity, and behavioral endpoint being measured should be important factors to consider when studying pain-depressed behavior.

1.7. Opioids in pain management

While opioids have therapeutic effects, adverse effects usually make them less than favorable. There are three main therapeutic effects of opioids, and they are (1) analgesia, (2) anti-diarrheal, and (3) cough suppression. As analgesics, opioids have been effective to treat acute and chronic pain, but the use of opioids for chronic non-cancer pain (CNCP) has been more controversial for reasons such as dependence, tolerance, and addiction (Rosenblum et al., 2008). A main reason for this are the long-term effects that opioids may have; however, many studies have shown that opioids such as fentanyl, morphine, and oxycodone have been more effective than placebo to manage different types of CNCP (e.g. low-back pain, neuropathic pain, fibromyalgia) (Furlan, 2006; Kalso et al., 2004). For instance, in a randomized, double-blind, placebo-controlled study (Watson et al., 2003), the effects of oxycodone (OxyContin) versus active placebo were studied to relieve painful diabetic neuropathy (PDN) in patients. The results showed that compared to active placebo, patients that received oxycodone had significantly lower pain intensity (VAS), pain relief (NRS), and improvement in quality of life (QOL) (i.e. physical functioning). As another example, in a randomized, double-blind study (Carpi et

al., 2020), the effects of intrathecal morphine vs ketamine were studied on post-operative analgesic effects in patients that had an abdominal hysterectomy. Results determined that compared to ketamine, patients that received morphine reported more pain relief because they had lower pain scores generated by the numerical rating scale (NRS) at 12-hours post-surgery. In another study (Liu et al., 2021), the low-efficacy opioid nalbuphine was studied in a randomized, double-blind, placebo-controlled study on post-operative analgesic effects in patients that had an abdominal cholecystectomy. The results showed that compared to placebo, patients that received nalbuphine reported lower pain intensity scores within 24-hours by the visual analog scale (VAS) and reported greater sleep quality. A second main therapeutic use of opioids is their use as antidiarrheals. Diarrhea, defined by an increase in intestinal motility, affects people for reasons such as viral or bacterial infections, or more serious causes such as irritable bowel syndrome (IBS). In either case, diarrhea can cause dangerous dehydration and loss of electrolytes. Loperamide, a peripheral opioid that is restricted from the central nervous system (CNS), is often used to treat diarrhea with the overall goal to slow gastrointestinal (GI) motility in order to reduce stool frequency (Schiller, 2017). The third therapeutic use of opioids is to suppress cough. While coughing is a useful physiological mechanism that allows for the clearance of the respiratory passage, excessive coughing, such as during a sickness, can become bothersome to disrupt sleep and rest. Opioids have long been used for cough suppression (e.g. heroin was marketed as a non-addictive cough suppressant) for their activation of MORs in the brain stem nuclei in the cough reflex pathway (Yaksh and Wallace, 2017). Codeine is naturally found in the opium poppy (thus an opiate), and studies have shown its effectiveness to decrease cough frequency

and severity and improvement of quality of life when compared to placebo (Yancy et al., 2013).

While the above lists therapeutic uses of opioids, this is not to say that they are without adverse effects. Nearly every study has data to show the main side effects reported and tolerated by patients and they include (in a non-specific order): constipation, sedation, pruritus (itching), tolerance, potential addiction, and respiratory effects. Some will be described below, and all these side effects are usually reported to a greater capacity in the opioid treated group vs the placebo group. The side effects are of main concern because the overall goal of opioid therapy, for instance, as analgesics, is to treat pain without adverse effects. For example, physical impairment induced by opioid effects such as sedation/somnolence, dizziness, and muscle rigidity are main adverse effects produced by high-efficacy opioids such as fentanyl, morphine, and oxycodone. While tolerance to these side effects can develop over time, the tolerability to these adverse events is often low, which can prevent the continued use of opioids (Benyamin et al., 2008). Constipation, or the infrequency of stool passage, is usually one of the most common reported side effects, and a reason why patients opt/drop out of clinical trials (Moore and McQuay, 2005). High-efficacy opioids are known to have addicting or rewarding properties, mechanistically through activation of MORs in the mesolimbic dopaminergic system (Gracely, 2013). Reports suggest that in clinical trials, “drug craving” is often asked in the inventory of questions about the addicting properties of opioids. However, reports also suggest that clinical trials are often not properly designed to ask questions regarding addiction, and interpreting these results is more difficult in patients with chronic pain (Kalso et al., 2004). While the side effects reported with the use

of opioids are of great concern, it is imperative to remember that these reports are often with the use of high-efficacy opioids (e.g. fentanyl, morphine, oxycodone), and that lower efficacy opioids (e.g. buprenorphine, nalbuphine) have a lesser risk of developing these side effects (White et al., 2018; Zeng et al., 2015). This is the reason why continued research into low-efficacy opioids is important for the treatment of pain.

1.8. Neurobiology and mechanisms of pain

The simplified three-neuron circuit for the transmission of pain information from the periphery to the cortex (the ascending pain pathway, Steps 1-5) and from cortex to the spinal cord (the descending pain pathway, Steps 6-11) is as shown in **Figure 1.8**. Step 1 begins in the periphery (e.g. skin, muscle, viscera), where the initiation, inhibition, or transduction of pain signals will be mediated by receptors or channels such as acid sensing ion channels (ASICs) which are responsive to IP lactic acid. Step 2 is the activation of one of the three classes of primary afferent neurons (PAN), which have cell bodies located in the dorsal root ganglion (DRG) as described in **Table 1.8.1.** PANs are pseudounipolar neurons with a main axon that branches in the DRG to send one projection to the periphery and a second projection to the spinal cord. The peripheral terminal is where the channels or receptors are located for detection of noxious stimuli and generation of action potentials, and in Step 3 the centrally directed branch of the PAN projects to the spinal cord. Step 4 involves spinothalamic neurons with cell bodies in the spinal dorsal horn that receive input from the PANs and project from the spinal cord up to midbrain areas such as the thalamus. Thalamocortical neurons with their cell bodies in

thalamus receive this information and then project to higher cortex brain areas (Step 5) such as the somatosensory cortex (SS-I).

Table 1.8.1: Peripheral afferent neurons (PANs)

Class	Cell body size	Myelinated	Speed	Modality
A-beta	Big	Yes	Fast	Mechanical intensity (not nociceptors)
A-delta	Medium	Yes	Medium	Thermal, mechanical, chemical intensity (some are nociceptors)
C	Small	No	Slow	Thermal, mechanical, chemical intensity (many are nociceptors)

Pain information received by the cortex is then relayed to other higher brain areas such as the anterior cingulate cortex (ACC), prefrontal cortex (PFC), and in Step 6, this information is relayed to neurons with their cell bodies in the amygdala in a “top-down” manner. In Step 7, these amygdalar neurons project to regions that include periaqueductal grey (PAG) in the brainstem. Step 8 involves the activation of opioid neurons in the PAG, and this activation of the opioid neuron can further act on the rostroventral medulla (RVM) in two ways. First, as seen in Step 9, opioid neurons can indirectly activate RVM OFF-cells by releasing endogenous opioid peptides that bind and activate MORs located on inhibitory GABA neurons, inhibiting the GABA neurons, and disinhibiting OFF-cells. These OFF cells then project to the spinal cord (Step 10) to inhibit nociceptive input and motor output in response to noxious stimuli (Step 3). Step 11 involves the direct inhibition of ON-cells in the RVM by opioid neurons to inhibit transmission to the spinal cord (Step 3). As seen in **Figure 1.8**, MOR are located in many locations both in the ascending and descending pathway. For instance, the localization

of MORs on the PAN projections near the periphery are important for the ability of MOR agonists to decrease overall pain signaling. They do this by decreasing cell signaling (as previously described in **section 1.2**), which in turn inhibits pre- and post-synaptic transmission in the dorsal horn of the spinal cord and activates the descending pain pathway through disinhibition of OFF-cells and inhibition of ON-cells in the RVM.

In **Chapters 4** and **5** of this dissertation, IP lactic acid was used as the acute pain stimulus. Therefore, a brief mechanistic explanation is warranted on IP acid and its effects on ascending pain system. Specifically, injection of acid in the peritoneal cavity causes hydrogen (H^+) ions to open acid-sensing ion channels (ASICs) and transient receptor potential vanilloid 1 (TRPV1) receptor located on the C-fibers of the PAN in the peritoneal cavity. ASICs and TRPV1 receptors are cation channels, and their activation allows sodium (Na^+) ions to enter the cell, which causes a depolarization of the cell to generate an action potential (AP). This action potential is transmitted from the peripherally along the axon to the spinal cord, where it promotes a release of excitatory neurotransmitters (e.g. glutamate) at the terminal end of the C-fibers in the dorsal horn of the spinal cord and initiates activation of the ascending pain pathway described in **Figure 1.8**.

In **Chapter 6** of this dissertation, three different types of pain stimuli were studied, and they are (1) intraplanar injection of complete Freund's Adjuvant (CFA), (2) laparotomy, and (3) the spared nerve injury (SNI); therefore, the mechanism of each will be further explained. Intraplanar CFA is a model that produces localized paw inflammation due to a mixture of heat, swelling, redness, and pain which is induced by heat-killed mycobacterium that triggers an immune response without direct tissue damage. The heat, swelling, and redness of the paw are all due to increased blood flow

and leaky vessel walls, which allows immune cell infiltration from the blood vessels into the tissue (Patil et al., 2019). This causes immune cells to the release inflammatory mediators which induce pain, and their net effect is (1) to cause minimal direct activation of the nociceptor, and (2) maximally cause primary sensitization of the nociceptor either in a direct or indirect fashion. Injection of CFA allows for the invasion of external bacteria, and those are referred to "Pathogen Associated Molecular Pattern" (PAMP) molecules. This is because lipopolysaccharide (a PAMP) is found in the walls of the heat-killed bacteria, and thus, triggers an immune response. Laparotomy is a model of post-surgical pain induced by an incision through the skin and visceral muscle followed by exposure and manipulation of the internal organs. This also causes (1) direct nociceptor activation due to the incision of the cutaneous and visceral tissue, and (2) inflammation that leads to primary sensitization of the nociceptor (Brennan, 2011). In this model, "Damage Associated Molecular Pattern" (DAMP) molecules, which are intracellular molecules that are usually not in the extracellular environment, are released due to the damaged and ruptured cells of the incised tissue, and because of damage to the skin, PAMP molecules from the invading external bacteria are released and their net effect is to trigger an immune response.

Examples of inflammatory mediators involved include protons that act on Acid Sensing Ion Channels (ASICs) and Transient Receptor Potential Vanilloid 1 (TRPV1) channels, adenosine triphosphate (ATP) that acts on P2Y receptors, bradykinin that binds B2 receptors, and increases in arachidonic acid metabolites such as prostaglandin (PGE2) which binds the EP2 receptor (Ringkamp et al., 2013). Cytokines are also involved in the inflammatory cascade and some examples are interleukin-1β (IL-1β),

interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) that are released from cells such as macrophages and are involved in the regulation of inflammation, neutrophils, endotoxins, the release of reactive oxygen species (ROS), and nerve growth factors (NGF) (Dawes et al., 2006; Pogatzki-Zahn et al., 2018; Ringkamp et al., 2013; Woolf et al., 1997) . The net effect of the indirect or direct release and activation of these mediators leads to a phenomenon that is referred to “peripheral sensitization” and this refers to changes in the sensitivity of the peripheral terminals of the nociceptor due to events such as the release of inflammatory mediators or increased membrane excitability (Baccei and Fitzgerald, 2013; von Hehn et al., 2012).

SNI is a model of mononeuropathy that is induced by directly damaging the nociceptor by ligating and cutting two out of three of the branches of the sciatic nerve (the tibial and common peroneal, leaving the sural nerve intact). This causes (1) direct nociceptor activation due to the incision to the cutaneous tissue, (2) minimal primary sensitization of the nociceptor due to inflammation at the surgical site, and (3) maximal secondary sensitization of the nociceptive circuitry. This phenomenon is also referred to as “central sensitization,” which refers to changes in the nociceptor terminal located in the dorsal horn of the spinal cord that leads to spontaneous neuronal activity, and recruitment of peripheral afferent neurons (PANs) that are not normally involved in mediating pain such as Aβ fibers (Latremoliere and Woolf, 2009; von Hehn et al., 2012). Two main mediators involved in central sensitization are (1) the continuous activation of excitatory inputs and spinal release of excitatory neurotransmitters such as glutamate that acts on N-methyl-D-aspartate receptors (NMDARs), and (2) reduced spinal release of inhibitory neurotransmitters such as γ-Aminobutyric acid (GABA) and glycine (Baccei and

Fitzgerald, 2013; Latremoliere and Woolf, 2009). The mechanism of action and duration of each stimuli described can be simplified as shown in **Table 1.8.2**., where an ordinal number system was implemented to attempt to describe the intensity of the effect produced where 1 = minimal effect, 2 = medium effect, and 3 = maximal effect.

Table 1.8.2: "Pain" stimuli mechanism and duration of action

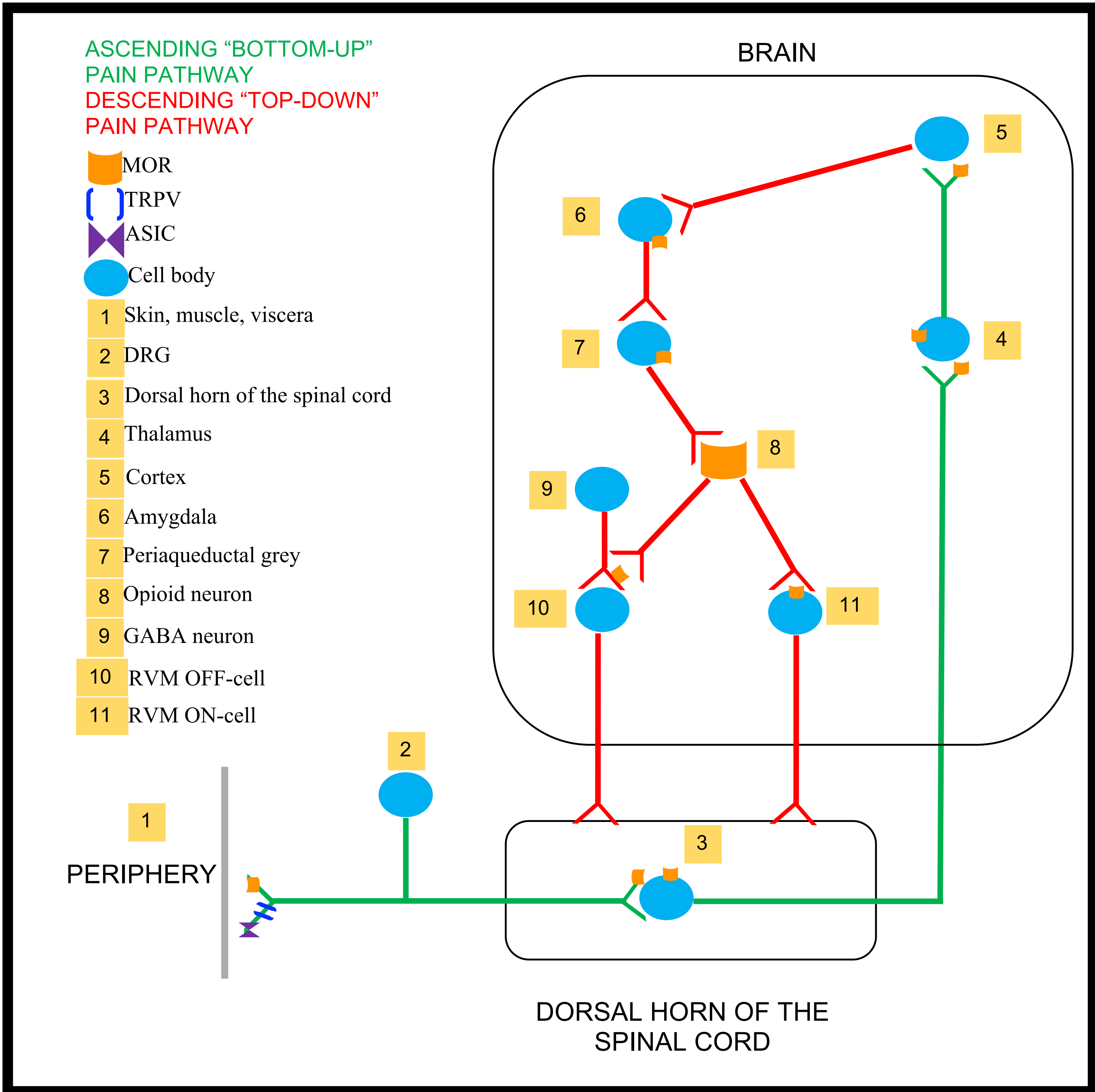
Pain stimulus	Direct nociceptor activation	Inflammation that leads to sensitized nociceptor	Damaged nociceptor that leads to secondary nociceptor sensitization	Duration of action
IP acid	3	2	1	1-2 hours
Laparotomy	3	3	1	Hours to days
Ipl CFA	1	3	1	Days to weeks
SNI	3	1	3	Months to permanent

1.9. Sex effects in opioid analgesia and pain

In 2015, the National Institutes of Health (NIH) laid out guidelines for including both male and female subjects in preclinical research (Miller et al., 2017). In accordance with this, the studies presented in this dissertation always included equal numbers of males and females along with a data analysis strategy that evaluated data pooled across both sexes and segregated by sex (Diester et al., 2019). The evaluation of sex differences was not a primary goal of our studies, and studies were not powered *a priori* to detect sex differences; however, our experimental design and data analysis strategy allowed us to conduct *post hoc* power analyses that could be used to investigate sex differences in more detail. Epidemiological studies of human pain research suggest that sex differences may be relevant. There is evidence to suggest sex differences in opioid analgesia; for example, reports suggest that, in studies of post-operative pain, women report less intake

of opioids than men, although this may be due to lower tolerance of side effects in women rather than to higher sensitivity to analgesia (Dahan et al., 2008; Fillingim and Gear, 2004). Additionally, women report more severe levels of pain, report pain more frequently, and have pain that lasts longer in duration than men, although this may be due to the fact that women are more comfortable or more willing to report pain and seek help (Greenspan and Traub, 2013). While sex as a biological variable was not a main dependent measure in the work presented in this dissertation, sex differences were studied and followed-up on when determined and will be discussed in further detail in each of the following chapters.

Figure 1.8. Ascending and descending pain pathway



1.10. Introduction to data chapters

The goal of this project was to study the role of MOR ligand efficacy in treatment of pain-depressed behavior. This was accomplished in three parts.

Part I: MOR efficacy as a determinant of locomotor activity. This was studied in **Chapters 2** and **3** of this dissertation, and the goal was to study the role of opioid efficacy in stimulating horizontal locomotor activity by testing 1) clinically available single-molecule opioids, 2) fentanyl/naltrexone mixtures, and 3) novel low-efficacy MOR selective opioids.

> **Chapter Two:** Investigated the effects of (1) clinically available single-molecule [listed from high- to low- MOR efficacy] methadone, fentanyl, morphine, hydrocodone, buprenorphine, nalbuphine, and naltrexone, (2) fentanyl/naltrexone mixtures [listed from high- to low- MOR efficacy] 100:1, 56:1, 32:1, 10:1, and 3.2:1, (3) time-course of these drugs over a 60-minute period, and (4) antagonism studies to determine receptor mechanism of action.

> **Chapter Three:** Investigated the effects of (1) novel single-molecule opioids [listed from high- to low- MOR efficacy] Tianeptine, DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, DC-01-76.1, EG-1-203, and EG-1-230, (2) time-course of the drugs over a 60-minute period, and (3) antagonism studies to determine receptor mechanism of action.

Part II: MOR efficacy as a determinant of antinociception in an assay of acute pain-depressed behavior. This was studied in **Chapters 4** and **5**, and the goal was to investigate the role of opioid efficacy to alleviate pain-depressed behavior induced by intraperitoneal (IP) lactic acid as an acute pain stimulus.

Chapter Four: Investigated the (1) validation of climbing behavior in mice in a four-step process, (2) the effects of clinically available single-molecule [listed from high- to low- MOR efficacy] fentanyl, buprenorphine, naltrexone (2) fentanyl/naltrexone mixtures [listed from high- to low- MOR efficacy] 10:1, 3.2:1, and 1:1 in the absence and presence of the pain stimulus.

Chapter Five: Investigated the (1) effects of novel single-molecule opioids [listed from high- to low- MOR efficacy] DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, DC-01-76.1, EG-1-203, and EG-1-230 in the absence and presence of the pain stimulus in the locomotor+barrier assay.

Part II: Expression of chronic pain-depressed behavior. This was studied in **Chapter 6** and the goal was to investigate the duration and magnitude of three different experimental chronic pain manipulations in an assay of pain-depressed behavior.

Chapter Six: Investigated the effects of three different chronic pain states in the locomotor + barrier behavioral assay. The pain states were (1) complete Freund's

Adjuvant (CFA) as a model of inflammation, (2) laparotomy as a model of post-surgical pain, and (3) spared nerve injury (SNI) as a mononeuropathy model.

Chapter Two

Role of Efficacy as a Determinant of Locomotor Activity in Male and Female Mice

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2.1. Introduction

Morphine and other mu opioid receptor (MOR) agonists produce a wide range of physiologic and behavioral effects that include both therapeutically useful effects like analgesia and undesirable effects that include disrupted motor function (Yaksh and Wallace, 2017). Mice are commonly used in preclinical drug development studies, and one MOR agonist effect in mice is a stimulation of horizontal locomotor activity (Frischknecht et al., 1983; Michael-Titus et al., 1989; Narita et al., 1993; Osborn et al., 2010; Raehal et al., 2005; Varshneya et al., 2019). Opioid induced locomotor activation in mice is one manifestation of MOR-mediated motor disruption, and as such, it can be considered an undesirable opioid effect. Additionally, opioid induced locomotor stimulation involves activation of the mesolimbic dopamine system and serves as one behavioral consequence of enhanced mesolimbic dopamine signaling (Botz-Zapp et al., 2021; Chefer et al., 2003; Funada et al., 1993; Severino et al., 2020; Urs and Caron, 2014; Walters et al., 2005). Lastly, locomotor activation is an unconditioned behavioral effect that requires no prior training for its expression, and it can be continuously and quantitatively measured in commercially available locomotor-activity chambers (Chakraborty et al., 2021; Raehal et al., 2005; Varshneya et al., 2019). These features make opioid-induced locomotor activation useful as an endpoint for early evaluation of the in vivo potency, effectiveness, and time course of novel opioids. The utility of opioid-

induced locomotor activation as a preclinical endpoint in drug evaluation would be further enhanced by clarification of its efficacy requirement relative to other opioid effects in mice and in other in vitro and in vivo test systems.

Accordingly, the goal of the present study was to evaluate the efficacy requirements for opioid-induced locomotor activation in male and female mice. Two parallel sets of studies were conducted. First, dose-effect curves were determined for a panel of eight MOR ligands with a range of maximal effects to stimulate GTPγS binding as an in vitro measure of relative MOR efficacy (Selley et al., 1998; Thompson et al., 2004; Thomsen et al., 2014; Yuan et al., 2013). Second, dose-effect curves were also determined for a panel of drug mixtures composed of the high-efficacy MOR agonist fentanyl and antagonist naltrexone. We have previously shown that the fixed proportion of fentanyl to naltrexone in fentanyl/naltrexone mixtures can be precisely manipulated to yield mixtures with graded maximal effects in both in vitro assays of ligand-stimulated GTPγS binding and in vivo assays across multiple endpoints in multiple species of test subject (Cornelissen et al., 2018; Schwienteck et al., 2019; Selley et al., 2021). Additionally, fentanyl/naltrexone mixtures can be used to identify the effective proportion of fentanyl sufficient to produce 50% of the maximum effect of fentanyl alone (defined as the EP50 value) as a metric of the efficacy requirement for any in vitro and in vivo MOR mediated effect (Cornelissen et al., 2018; Schwienteck et al., 2019; Selley et al., 2021). Prevailing evidence suggests that locomotor activation may have a higher efficacy requirement than some other opioid effects, such as thermal antinociception in mice (Chakraborty et al., 2021; Varshneya et al., 2021, 2019). As a result, we predicted that

the EP50 value for locomotor activation in mice would be high relative to EP50 values we have determined previously for other opioid effects in mice, rats, and rhesus monkeys.

2.2. Methods

2.2.1. Animals

Subjects were male and female ICR mice (Envigo, Frederick, MD) that were 6–8 weeks old upon arrival to the laboratory. Males weighed 27–50g and females weighed 22–50 g throughout the study. Mice were housed in same-sex, littermate groups in cages with corncob bedding (Envigo), a “nestlet” composed of pressed cotton (Ancare, Bellmore, NY), a cardboard tube for enrichment, and ad libitum access to food (Teklad LM-485 Mouse/Rat Diet; Envigo). Cages were mounted in a RAIR HD Ventilated Rack (Laboratory Products, Seaford, DE) in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed during the light phase of the daily light/dark cycle beginning 1 week after arrival at the laboratory. Ethical animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (Protocol #AD10001093) and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

2.2.2. Apparatus

Horizontal locomotor activity was assessed during 60-minute sessions in test boxes (16.8 × 12.7 cm2 floor area × 12.7 cm high) housed in sound-attenuating chambers

(Med Associates, St. Albans, VT) and located in a procedure room separate from the housing room. Each box had black plexiglass walls, a clear plexiglass ceiling equipped with a house light, bar floors, and six photobeams arranged at 3-cm intervals across the long wall and 1 cm above the floor. Beam breaks were monitored by a microprocessor operating Med Associates software. The primary dependent variable was the total number of beam breaks, excluding consecutive interruptions of the same beam, during the 60-minute session.

2.2.3. Pharmacological Procedure

The primary goal of the study was to test a range of MOR ligands that varied in their relative efficacy at the MOR as quantified by maximum agonist-stimulated GTPɣS binding in Chinese hamster ovary (CHO) cells expressing the mouse or human MOR in previously published studies (Obeng et al., 2018; Selley et al., 2021, 1998; Thompson et al., 2004). This was accomplished by testing two different categories of treatments. First, a range of eight different single-molecule MOR ligands was tested. These drugs and their associated dose ranges were as follows (listed from highest to lowest maximum effect (Emax) in studies of agonist-stimulated GTPɣS binding): methadone, 0.32–32 mg/kg (Middaugh and Zemp, 1976), fentanyl, 0.032–3.2 mg/kg (Varshneya et al., 2019), morphine, 1.0–100 mg/kg (Loggi et al., 1991), hydrocodone, 1.0–100 mg/kg (Jacob et al., 2017), buprenorphine, 0.01 – 3.2 mg/kg (Cowan et al., 1977), nalbuphine, 0.32–32 mg/kg (Patrick et al., 1999), NAQ (17-Cyclopropylmethyl-3,14b- dihydroxy-4,5a-epoxy-6a-[(30-isoquinolyl)acetamido]-morphinan), 1.0–100 mg/kg (Zhang et al., 2014), and naltrexone, 0.1–3.2 mg/kg (Castellano and Puglisi-Allegra, 1982). Nalbuphine, NAQ, and naltrexone produced little or no locomotor stimulation across the dose-range tested, so each of these

drugs were further evaluated for effectiveness to block locomotor activation by 10 mg/kg of morphine. The second category of treatments consisted of a series of fixed-proportion fentanyl/naltrexone mixtures. In these mixtures, the proportion of fentanyl to naltrexone was fixed at a constant value for a given mixture (e.g., 1:1 fentanyl/naltrexone), and changes in the dose of one drug of the mixture were matched by equivalent changes in the other drug. We have reported previously that the net MOR efficacy of fentanyl/naltrexone mixtures can be precisely calibrated both in vitro and in vivo by adjusting the fentanyl proportion in the mixture, such that increasing fentanyl proportions result in increasing levels of MOR efficacy for the mixture. The present study compared effects of five different fentanyl/naltrexone mixtures ranging from 100:1 to 3.2:1 fentanyl/naltrexone. With two exceptions noted below, a different group of 12 mice (six females, six males) were used to test each drug or mixture, and we have previously presented a detailed rationale for this group size and sex allocation (Diester et al., 2019). For this study, cohorts of up to 36 mice were generally used at any one time to test three different drugs or mixtures, and mice in each cohort were randomly assigned to the different treatments. Within each group, test sessions were conducted twice a week with at least 48 hours between sessions. All mice received a vehicle control and all doses of the designated test drug or mixture, and dose order was randomized across mice using a Latin-square design. The experimenter was not blinded to treatment because data collection was automated by the Med Associates software. There were no exclusion criteria, and all data were included in final analysis. On test days, mice were brought to the procedure room at least 2 hours before session onset. After subcutaneous test-drug administration, mice were returned to their home cages for the 5-minute pretreatment

interval and then placed into the locomotor activity boxes at session onset. Doses for each drug or mixture were varied in 0.5 or 1.0 log-unit increments across a ≥10-fold dose range with the intent of progressing from low doses that produced little or no effect to high doses that produced maximal increases in locomotor activation for that drug. For nalbuphine, NAQ, and naltrexone, antagonism studies were conducted after completion of drug-alone studies in the same mice. Doses of the test drug were administered 10 minutes before 10 mg/kg of morphine, and locomotor sessions began 5 minutes after morphine administration. There were two exceptions to this general design. First, in the case of hydrocodone, only six of the original mice (three of each sex) were tested at the high dose of 100 mg/kg due to limited drug supply. Because a clear effect plateau had not been reached at this dose, more drug was acquired and a higher dose of 320 mg/kg of hydrocodone was tested in four other mice (two of each sex); however, all mice died, and further studies with hydrocodone were not pursued. Second, in the case of buprenorphine, the initial group was tested only up to a dose of 1.0 mg/kg due again to limited drug supply. Because a clear effect plateau had not been reached at this dose, more drug was acquired, and a higher dose of 3.2 mg/kg was tested in six other mice (three of each sex).

2.2.4. Data and Statistical Analysis

The primary dependent variable was the total number of beam breaks, excluding consecutive interruptions of the same beam, during each 60-minute session. These data were first analyzed within each drug or mixture to assess dose-dependent effects. Initial within-drug analysis proceeded in four phases as described previously for studies that include both females and males but are not intended a priori to detect sex differences

(Diester et al., 2019). First, because sex was not the primary variable of interest, pooled data from both females and males were analyzed by repeated-measures one-way ANOVA with dose as the single variable, and a significant ANOVA was followed by a Holm-Sidak post-hoc test to both (a) identify doses producing effects different from vehicle and (b) evaluate presence or absence of a significant difference between the highest doses to identify an effect plateau for Emax determination. For this and all other analyses described below, the criterion for significance was $P < 0.05$. Data for the highest doses of hydrocodone and buprenorphine were not included in the one-way ANOVA for these drugs because of the lower number of mice tested; rather, effects of these doses were compared with the next lower dose by t test (paired for hydrocodone, unpaired for buprenorphine). Second, data were segregated by sex and again submitted to repeated-measures one-way ANOVA followed by Holm-Sidak post-hoc test to assess dose-dependent effects within each sex. Third, male and female data were directly compared by two-way ANOVA with sex as a between-subjects factor and drug dose as a within-subjects factor. A significant sex × dose interaction was followed by a Holm-Sidak post-hoc test. These first three steps of data analysis were performed using GraphPad Prism 9.0 (La Jolla, CA). Lastly, the two-way ANOVA results were submitted to power analyses to calculate the Cohen's f effect size, achieved power ($1 - \beta$), and the total number of animals predicted as necessary to detect a significant effect of sex, dose, and the sex × dose interaction given the effect size, $\alpha = 0.05$, and power $(1 - \beta) = 0.8$ using the free statistical analysis program G*Power (Faul et al., 2007). Regarding the antagonism studies, data analysis was performed as described above in steps 1–3 with the exception that test drugs were evaluated for their effectiveness to decrease locomotor stimulant

effects of morphine. Taken together, this strategy for experimental design and data analysis is intended to treat sex as an important but secondary variable of interest and to provide exploratory power analysis that can guide future studies explicitly designed to explore sex as a biologic variable (Diester et al., 2019). Following this within-drug analysis, three additional types of analyses were conducted. First, the maximal effects of each drug or fentanyl/naltrexone mixture at any dose were compared, and these Emax values were considered to be different if 95% confidence limits did not overlap. Second, the Emax of each drug or mixture for locomotor stimulation was transformed to a percentage of the fentanyl-alone Emax (% Fent Max) using the equation (Test Drug Emax – Vehicle Baseline) / (Fentanyl Emax – Vehicle Baseline)) *100, where "Emax" was the maximum number of locomotor counts for a test drug or fentanyl at any dose, and "Baseline" was the number of counts after vehicle treatment in that group. Values for % Fent Max of each drug and mixture were then graphed as a function of previously published Emax values of each drug or mixture to stimulate GTPɣS binding in CHO cells expressing cloned MOR (Obeng et al., 2018; Selley et al., 2021, 1998; Thompson et al., 2004). Data for single-molecule ligands and fentanyl/naltrexone mixtures were submitted separately to linear regression analyses for the linear sections of their respective curves to identify the magnitudes of GTPɣS binding (95% CL) associated with 50% Fent Max. Values were considered to be statistically similar if 95% confidence limits overlapped, and we predicted that these values would be similar for both single-molecule MOR ligands and fentanyl/naltrexone mixtures. Lastly, data for the fentanyl/naltrexone mixtures were used to determine an EP50 value, defined as the proportion of fentanyl in the fentanyl/naltrexone mixture that produces an Emax equal to 50% of the fentanyl-alone

Emax. As we have described previously, the EP50 value determined from a series of fentanyl/naltrexone mixtures can be used to quantify the efficacy requirement for a given endpoint of MOR agonist induced effects, such that higher EP50 values indicate higher efficacy requirements. To calculate the EP50 value, the Emax of each mixture was again expressed as % Fent Max and graphed as a function of the fentanyl proportion for each mixture. These fentanyl proportion-Emax data were submitted to nonlinear regression to determine the EP50 (95% CL). This EP50 value for locomotor activity in mice determined in the present study was then compared with previously determined EP50 values for fentanyl/naltrexone mixtures to produce a range of other effects in previously published studies (Cornelissen et al., 2018; Schwienteck et al., 2019; Selley et al., 2021). EP50 values across endpoints were considered to be different if 95% confidence limits did not overlap.

2.2.5. Materials

(±) Methadone HCl, fentanyl HCl, morphine sulfate, hydrocodone bitartrate, buprenorphine HCl, nalbuphine HCl, and naltrexone HCl were all provided by the National Institute on Drug Abuse Drug Supply Program. 17-Cyclopropyl-methyl-3,14b-dihydroxy-4,5a-epoxy-6a-[(30-isoquinolyl) acetamido]-morphinan (NAQ) was synthesized by Dr. Yan Zhang (Virginia Commonwealth University). In addition to these single-molecule test drugs, five fentanyl/naltrexone (FENT/NTX) mixtures were tested with fentanyl-to-naltrexone proportions of 100:1, 56:1, 32:1. 10:1, and 3.2:1. All compounds were administered subcutaneously (SC) per body weight in volumes of 0.1–0.9 ml and

dissolved in sterile saline, except for NAQ, which was dissolved in 10% DMSO and 90% water.

2.3. Results

Figure 2.1 shows pooled data from both sexes for locomotor effects of all single-molecule opioids. One-way ANOVA results and Emax values for each drug are shown in **Table 2.1**, and **Fig. 2.2** shows the time course of effects produced by selected doses of each drug over the 60-minute session. Methadone, fentanyl, morphine, hydrocodone, buprenorphine, and nalbuphine produced dose-dependent and significant locomotor stimulation, whereas NAQ and naltrexone did not. Each drug was tested up to an effect plateau at which increasing doses failed to produce further significant increases in locomotion. Note that, for hydrocodone, a higher dose of 320 mg/kg was tested in a subset of four male and female mice, and all died in <30 minutes. No dose of any other drug produced lethality in any other mice. Emax values for methadone, fentanyl, morphine, hydrocodone, and buprenorphine were similar to each other and higher than for nalbuphine. Nalbuphine, NAQ, and naltrexone all produced a dose-dependent blockade of morphine-induced locomotor stimulation.

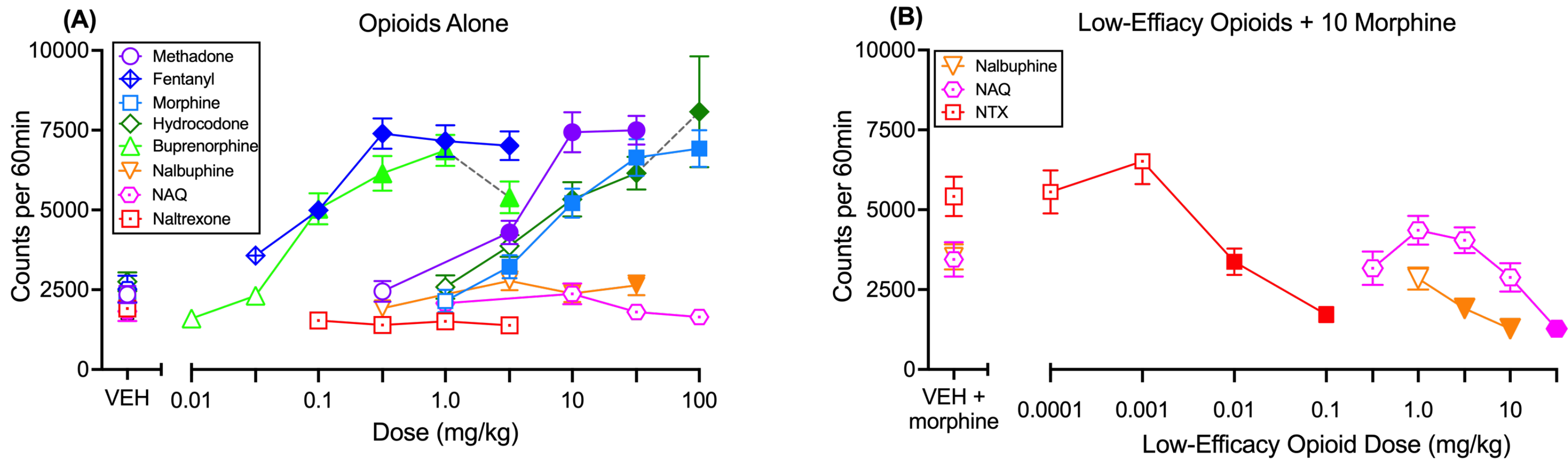


Figure 2.1: Locomotor activating effects of opioids with differing MOR efficacy. (A) Effects of opioids administered alone. (B) Effects of nalbuphine, NAQ and naltrexone administered as a pretreatment to 10 mg/kg morphine. Abscissae: Dose in mg/kg. Ordinates: Locomotor counts per 60 minutes. In general, all points show mean±S.E.M. for N = 12 mice, and filled symbols indicate a significant difference compared with vehicle within each drug as determined by repeated-measures one-way ANOVA followed by the Holm-Sidak post hoc test, $P < 0.05$. There were two exceptions. The dashed line to 3.2 mg/kg of buprenorphine indicates low sample size (N = 6) and a different cohort of mice for this dose, and the filled point indicates different from vehicle by unpaired t test. The dashed line at 100 mg/kg hydrocodone indicates low sample size (N = 6) but in the same cohort of mice for this dose, and the filled symbol indicates different from vehicle by paired t test. A different group of four male and female mice tested with a higher hydrocodone dose (320 mg/kg) all died, so further studies at this dose were not conducted, and these data are not included in the graph. Statistical results for Panel A are shown in Table 2.1. For Panel B, one-way ANOVA results were as follows. Nalbuphine: $F(1.64, 18.02)$ 5 14.42; P 5 0.0003; NAQ: $F(4.15, 45.68)$ 5 8.67; $P < 0.0001$; naltrexone: $F(2.59, 28.45)$ 5 27.35; $P < 0.0001$.

Table 2.1: One-way ANOVA results and Emax values for data shown in Fig. 2.1.A. Hydrocodone and buprenorphine data in this table do not include high doses due to different N.

Opioid	One-way ANOVA	Emax (95% CI)
Methadone	$F(2.89, 31.87) = 38.81$; $p<0.0001$	7500 (6505, 8495)
Fentanyl	$F(2.97, 32.63) = 26.94$; $p<0.0001$	7393 (6346, 8440)
Morphine	$F(2.76, 30.40) = 31.75$; $p<0.0001$	6925 (5662, 8188)
Hydrocodone	$F(2.08, 22.86) = 13.81$; $p=0.0001$	6153 (5025, 7280)
Buprenorphine	$F(2.58, 28.34) = 39.39$; $p<0.0001$	6867 (5802, 7933)
Nalbuphine	$F(3.45, 37.90) = 3.29$; $p=0.0254$	2639 (1954, 3324)
NAQ	$F(2.52, 27.67) = 2.06$; $p=0.1376$	-
Naltrexone	$F(2.41, 26.46) = 1.69$; $p=0.2001$	-

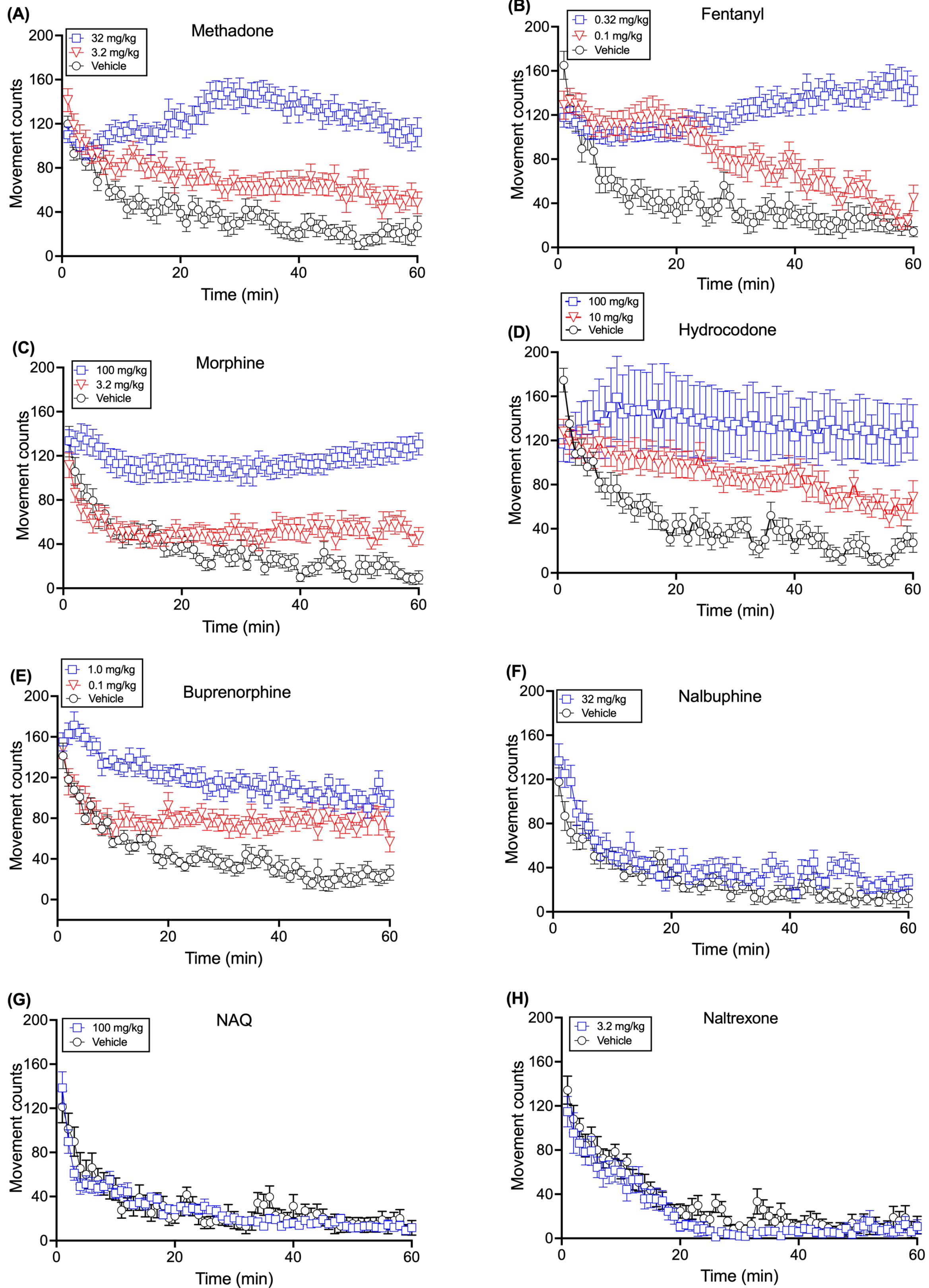


Figure 2.2: Time course of locomotor activating effects produced by opioids with differing MOR efficacy.

Figure 2.2: Time course of locomotor activating effects produced by opioids with differing MOR efficacy [figure legend]. Each panel shows time course data over a 60-minute session for a different drug. For most drugs, data are shown for vehicle, the lowest dose to significantly increase locomotion, and the Emax dose producing the highest level of locomotor activation. Nalbuphine, NAQ, and naltrexone show only data for vehicle and the highest dose tested. Abscissae: Time in min for the 60-minute session. Ordinates: movement counts over the 60-minute session. Each point shows mean±S.E.M. from 12 mice except the high dose for hydrocodone, which shows N = 6.

Figure 2.3 shows pooled data from both sexes for locomotor effects of the fentanyl/naltrexone mixtures. One-way ANOVA results and Emax values for each mixture are shown in **Table 2.2**. The 100:1, 56:1, 32:1, and 10:1 fentanyl/naltrexone mixtures produced dose-dependent and significant increases in locomotor activity, whereas the 3.2:1 mixture did not. The mixture with the highest fentanyl proportion (100:1) produced the highest Emax value, which was not significantly different from the Emax for fentanyl alone (see **Table 2.1**), and mixtures with progressively lower fentanyl proportions produced progressively lower Emax values.

Results in **Figures 2.1-2.3** indicate that, within boundaries described below, increasing MOR efficacy is associated with increasing locomotor activation in mice. The efficacy requirements for locomotor activation were quantified in two ways. First, **Figure 2.4** shows the relationship between (a) the Emax value of each single-molecule opioid and fentanyl/naltrexone mixture in the present study of locomotor activation and (b) the Emax value in prior studies of ligand-stimulated GTPɣS binding in CHO cells expressing cloned MOR. Drugs or mixtures with in vitro Emax values from 0% to approximately 50% of the DAMGO Emax produced graded increases in locomotor activity; however, further increases in the in vitro Emax values (with morphine, fentanyl, and methadone) did not produce further increases in locomotor activity. The mean (95% CL) magnitude of ligand-stimulated GTPɣS binding associated with a locomotor Emax equal to 50% of the fentanyl-alone Emax was similar for both single-molecule opioids [30.8 (25.1-37.2)] and fentanyl/naltrexone mixtures [29.2 (10.2-41.9)]. The mean (95% CL) slopes of the regressions were also similar [2.43 (1.66-3.21) for single-molecule opioids; 1.91 (0.81-3.01) for fentanyl/naltrexone mixtures].

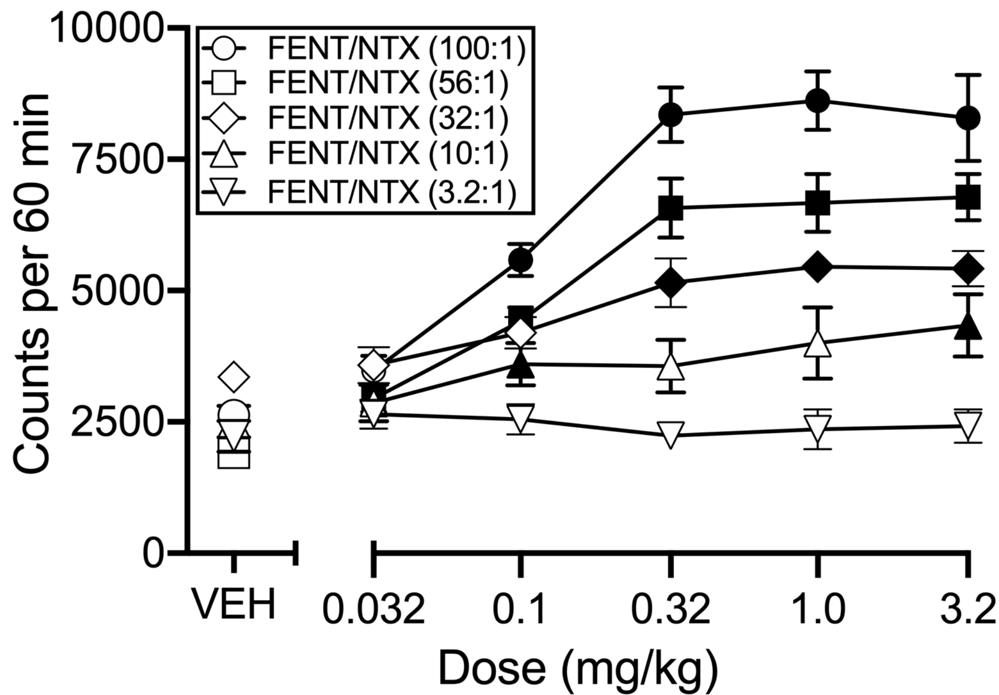


Figure 2.3: Locomotor activating effects of fentanyl/naltrexone mixtures. Abscissa: Dose fentanyl in mg/kg. The naltrexone dose was proportional to the fentanyl dose as indicated by fixed fentanyl/naltrexone (FENT/NTX) proportions for each mixture. Ordinate: Locomotor counts per 60 minutes. All points show mean±S.E.M. for N 5 = 12 mice, and filled symbols indicate a significant difference compared with vehicle within each mixture as determined by repeated-measures one-way ANOVA followed by the Holm-Sidak post hoc test, P < 0.05. Statistical results are shown in Table 2.

Table 2.2: One-way ANOVA results and Emax values for fentanyl/naltrexone mixtures in Fig. 2.3.

Fentanyl/naltrexone mixture	One-way ANOVA	Emax (95% CI)
100:1	$F(2.39, 26.37) = 40.84$; $p<0.0001$	8615 (7386, 9843)
56:1	$F(2.16, 23.79) = 36.28$; $p<0.0001$	6777 (5805, 7748)
32:1	$F(3.27, 35.94) = 14.06$; $p<0.0001$	5458 (4854, 6061)
10:1	$F(2.68, 29.43) = 6.60$; $p=0.0021$	4338 (3033, 5643)
3.2:1	$F(3.82, 41.99) = 0.76$; $p=0.5506$	-

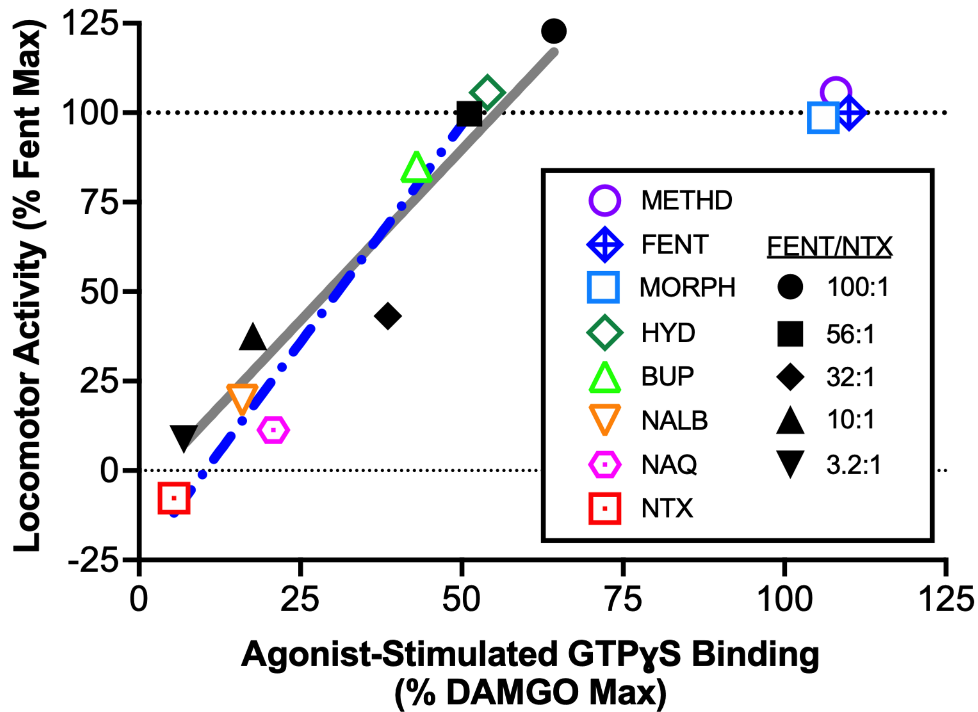


Fig. 2.4: Relationship between MOR agonist and fentanyl/naltrexone mixture effects on in vitro activation of GTPγS binding and in vivo locomotor stimulation. Abscissa: Emax for each drug or mixture to stimulate GTPγS binding in CHO cells expressing cloned MOR from previously published studies (see text for citations). Data are expressed as a percentage of the maximum effect of the high-efficacy MOR agonist DAMGO, which was included as a standard in each study. Ordinate: Emax for each drug or mixture to stimulate locomotor activity in the present study. Data are expressed as a percentage of the maximum effect produced by fentanyl. The blue dotted line shows linear regression for single-molecule opioids on the linear portion of the curve (morphine, fentanyl, and methadone excluded). The gray solid line shows linear regression for the fentanyl/naltrexone mixtures. Abbreviations: BUP, buprenorphine; HYD, hydrocodone; METHD, methadone; MORPH, morphine, FETN, fentanyl; NALB, nalbuphine; NTX, naltrexone.

Second, **Figure 2.5** shows determination of the locomotor EP50 value, with EP50 value defined as the fentanyl proportion of the fentanyl/naltrexone mixture sufficient to produce an Emax equal to 50% of the fentanyl-alone Emax. In so far as the EP50 value serves as a metric of the efficacy requirement for a given MOR-mediated effect, these results indicate that the efficacy requirement determined in the present study for locomotor activation in mice [EP50 (95% CL) = 18.6 (11.4-38.0)] is higher than in assays of opioid discrimination in rats or thermal antinociception in mice, similar to thermal antinociception in rats and rhesus monkeys, and lower than for stimulation of GTPɣS binding in CHO cells expressing cloned MOR.

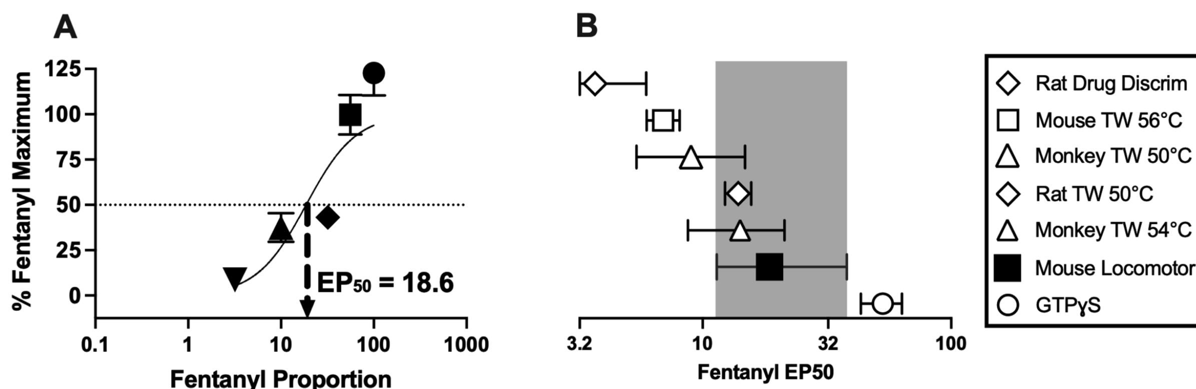


Figure 2.5: EP50 values as a metric of efficacy requirement for different effects produced by fentanyl/naltrexone mixtures. (A) Abscissa: Fentanyl proportion in different fentanyl/naltrexone mixtures. Ordinate: Maximum locomotor activating effects of each mixture expressed as a percentage of the fentanyl-alone maximum. Each point shows mean±S.E.M. for 12 mice, and nonlinear regression was used to calculate the EP50, which is defined as the fentanyl proportion in a fentanyl/naltrexone mixture that would produce a maximum effect equal to 50% of the fentanyl-alone maximum effect. (B) EP50 value (95% CL) for locomotor activation in the present study relative to EP50 values for fentanyl/naltrexone mixtures determined in previous studies using various behavioral endpoints in mice, rats, and rhesus monkeys or in the in vitro assay of ligand-stimulated GTPɣS binding in CHO cells expressing cloned MOR. Assays with EP50 values to the left of the shaded box have lower efficacy requirements than locomotor activation, whereas points to the right of the shaded box have higher efficacy requirements than locomotor activation. Abbreviations: Drug Discrim, drug discrimination; TW, warm-water tail-withdrawal with water temperature specified in C.

Although the present study was not intended to rigorously evaluate sex differences in opioid effects, it did include both male and female subjects and did permit two-way dose x sex ANOVAs and subsequent post hoc power analysis for preliminary evaluation of sex as determinant of opioid effects. Results of these analyses are shown in **Table 2.3** (main effects of dose), **Table 2.4** (main effects of sex) and **Table 2.5** (dose x sex interaction), which show two-way ANOVA results, Cohen's effect size, current power, and projected sample size to achieve power ≥0.8 for all treatments. These analyses confirmed a main effect of dose for most single-molecule opioids and fentanyl/naltrexone mixtures, but not for NAQ, naltrexone, or the 3.2:1 fentanyl/naltrexone mixture. Main effects of sex or dose x sex interactions were rare, and in general, post hoc power analysis indicated that power and associated sample sizes were too low to detect sex differences. Nonetheless, there were main effects of sex for the lowest two fentanyl/naltrexone mixtures (10:1 and 3.2:1) as shown in **Figure 2.6**, with males showing higher locomotion regardless of dose, including after vehicle treatment. There was also a significant dose × sex interaction for both the 32:1 fentanyl/naltrexone mixture and for hydrocodone, but for both treatments, post-hoc analysis did not identify a significant effect of sex at any dose of the mixture as shown in **Figure 2.7.** Thus, even these significant sex effects provided weak evidence for a role of sex as a determinant of opioid-induced hyperactivity.

Table 2.4: Two-way ANOVA results with power analysis of main effect of sex.

Opioid/Mixture	F statistic; p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Methadone	F(2.820, 28.20) = 38.75; p<0.0001	1.965	1.0	10
Fentanyl	F(3.337, 33.37) = 29.25; p<0.0001	1.710	0.975	10
Morphine	F(2.715, 27.15) = 29.89; p<0.0001	1.728	0.953	10
Hydrocodone	F(2.757, 27.57) = 18.95; p<0.0001	1.376	0.896	10
Buprenorphine	F(2.627, 26.27) = 38.49; p<0.0001	1.962	0.984	10
Nalbuphine	F(3.358, 33.58) = 3.18; p=0.032	0.564	0.205	55
NAQ	F(2.489, 24.89) = 1.92; p=0.159	0.439	0.144	90
Naltrexone	F(2.356, 23.56) = 1.56; p=0.229	0.396	0.122	>100
100:1	F(2.614, 26.14) = 44.61; p<0.0001	2.112	0.993	10
56:1	F(2.154, 21.54) = 33.20; p<0.0001	1.822	0.936	10
32:1	F(3.036, 30.36) = 16.39; p,0.0001	1.280	0.781	15
10:1	F(2.821, 28.21) = 6.856 p=0.002	0.828	0.373	30
3.2:1	F(3.515, 35.15) = 0.75; p=0.551	0.273	0.081	>100

Table 2.4: Two-way ANOVA results with power analysis of main effect of sex.

Opioid/Mixture	F statistic; p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Methadone	F(1, 10) = 0.42; p=0.534	0.124	0.068	>100
Fentanyl	F(1, 10) = 0.00; p=0.952	0.010	0.050	>100
Morphine	F(1, 10) = 1.77; p=0.213	0.299	0.156	90
Hydrocodone	F(1, 10) = 0.20; p=0.665	0.084	0.058	>100
Buprenorphine	F(1, 10) = 0.85; p=0.379	0.212	0.102	>100
Nalbuphine	F(1, 10) = 0.48; p=0.506	0.212	0.102	>100
NAQ	F(1, 10) = 0.04; p=0.845	0.055	0.053	>100
Naltrexone	F(1, 10) = 0.02; p=0.897	0.027	0.051	>100
100:1	F(1, 10) = 1.41; p=0.263	0.320	0.172	80
56:1	F(1, 10) = 0.00; p=0.977	0.007	0.050	>100
32:1	F(1, 10) = 0.96; p=0.351	0.359	0.203	65
10:1	F(1, 10) = 7.15; p=0.023	1.198	0.962	10
3.2:1	F(1, 10) = 12.66; p=0.005	1.073	0.917	10

Table 2.5: Two-way ANOVA results with power analysis of dose x sex interaction.

Opioid/Mixture	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Methadone	$F(4, 40) = 0.98; p=0.428$	0.313	0.282	35
Fentanyl	$F(5, 50) = 1.94; p=0.104$	0.441	0.607	20
Morphine	$F(5, 50) = 0.36; p=0.875$	0.189	0.133	75
Hydrocodone	$F(4, 40) = 5.09; p=0.002$	0.713	0.944	10
Buprenorphine	$F(5, 50) = 0.75; p=0.589$	0.274	0.248	40
Nalbuphine	$F(5, 50) = 0.62; p=0.689$	0.248	0.206	45
NAQ	$F(4, 40) = 0.30; p=0.877$	0.173	0.110	>100
Naltrexone	$F(4, 40) = 0.18; p=0.947$	0.135	0.085	>100
100:1	$F(5, 50) = 2.02; p=0.093$	0.449	0.626	20
56:1	$F(5, 50) = 0.07; p=0.997$	0.081	0.063	>100
32:1	$F(5, 50) = 2.82; p=0.025$	0.531	0.794	15
10:1	$F(5, 50) = 1.43; p=0.231$	0.378	0.460	25
3.2:1	$F(5, 50) = 0.79; p=0.564$	0.280	0.259	40

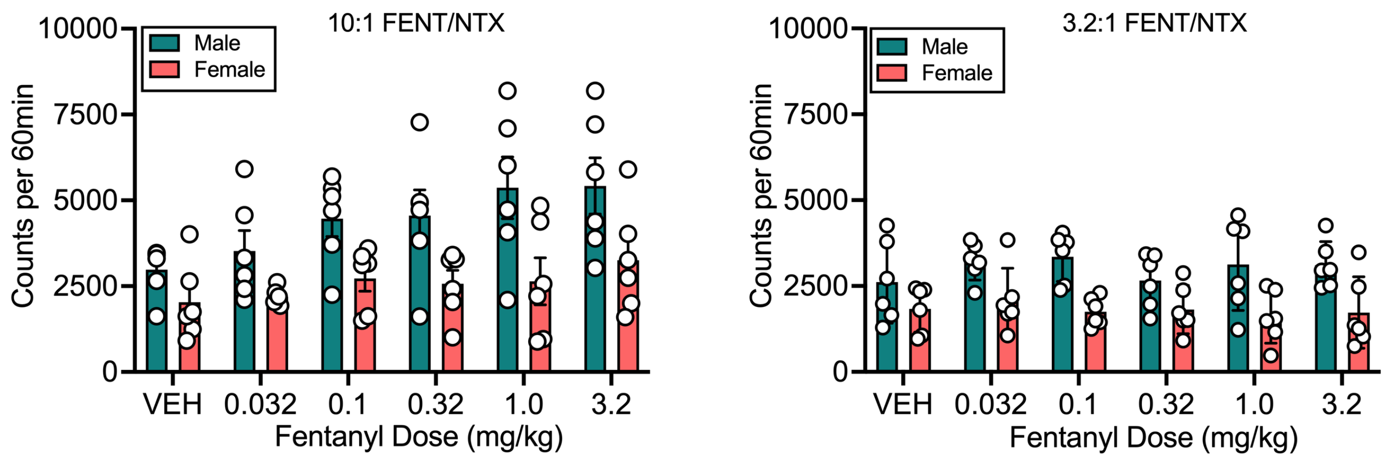
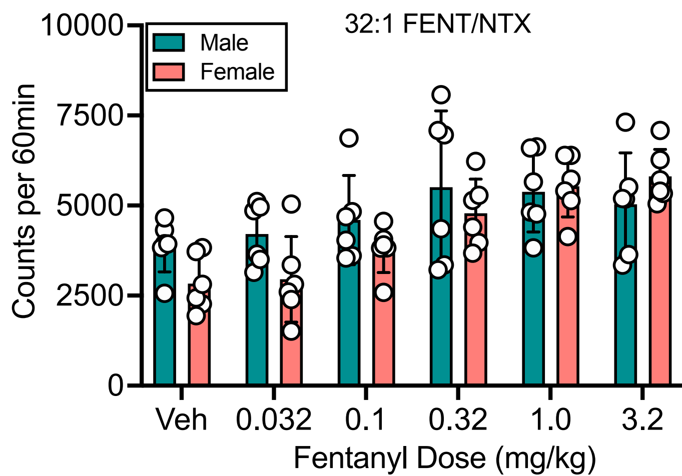


Figure 2.6: Significant main effect of sex. Abscissae: Dose fentanyl in mg/kg (VEH=vehicle). The naltrexone dose = fentanyl dose ÷ fentanyl proportion (10 in 1a, 3.2 in 1b). Ordinates: Locomotor counts per 60 minutes. Male and female data are shown in green and orange bars, respectively, and individual data are shown by points. Males had higher locomotor scores than females for these two treatments.



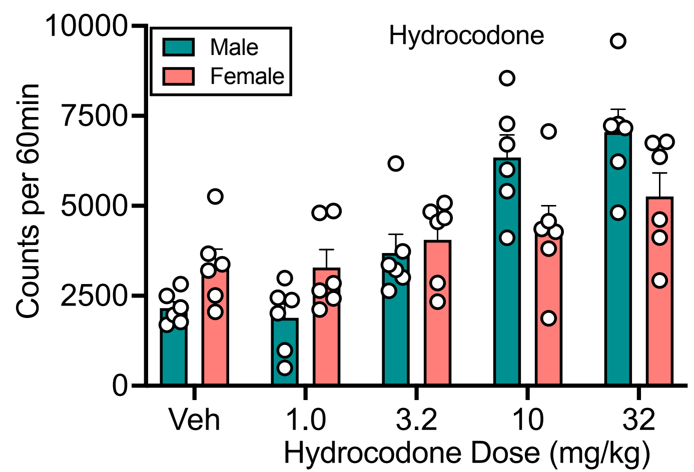


Figure 2.7: Significant dose x sex interaction. Abscissae: Dose in mg/kg (VEH=vehicle). For panel 2a, the abscissa shows fentanyl dose, and the naltrexone dose = fentanyl dose ÷ fentanyl proportion (32). Ordinates: Locomotor counts per 60 minutes. Male and female data are shown in green and orange bars, respectively, and individual data are shown by points. Post hoc analysis did not indicate a significant difference between males and females at any dose of either treatment.

2.4. Summary.

This study evaluated locomotor activation produced in mice by a panel of single-molecule opioids and fentanyl/naltrexone mixtures. There were three main findings. First, these results provide evidence for efficacy-dependent MOR agonist effects on maximal locomotor activation in mice. This finding suggests that in vivo assessment of mouse locomotor activity can serve as an efficient tool for in vivo stratification of the MOR efficacies of opioid ligands. Second, the apparent efficacy requirement for locomotor activation was relatively high in comparison with previously determined efficacy requirements for other in vivo opioid effects in mice, such as antinociception. To the degree that locomotor activation in mice is an undesirable sign of opioid-induced motor disruption, these findings suggest the potential for low-efficacy MOR ligands to produce effects of potential therapeutic benefit (e.g., thermal antinociception) with minimal motor disruption. Lastly, the present results provided weak evidence for sex differences in opioid-induced locomotor stimulation, but when differences were observed, locomotor activity was higher in males. These results could provide a foundation for future efforts to explore sex differences in opioid-induced locomotor activation.

Chapter Three

Role of Efficacy as a Determinant of Locomotor Activation by Mu Opioid Receptor (MOR) Ligands in Female and Male Mice. II. Effects of Novel MOR-Selective Opioids with a Range of MOR Efficacies

3.1. Introduction

Opioids that vary in their efficacy at MOR produce different maximal effects of hyperlocomotor activity in mice as discussed in **Chapter 2** of this dissertation. In **Chapter 2**, a series of clinically available single-molecule opioids and fentanyl/naltrexone mixtures were studied as two tools to manipulate efficacy at the mu-opioid receptor (MOR). In this Chapter, a third tool to manipulate MOR efficacy will be discussed, and that is the study of novel single-molecule opioids. As mentioned in the Introduction chapter of this dissertation [**Chapter 1; section 1.3**], efficacy and receptor selectivity are important pharmacodynamic variables. Specifically, the work presented in this dissertation has focused on studying low-efficacy opioids as potential useful analgesics; however, one constraint with current clinically available low-efficacy opioids (e.g. buprenorphine, nalbuphine) has been their poor selectivity at MOR (Gudin and Fudin, 2020; Pick et al., 1992). Thus, the study presented here aims to target the poor selectivity of current low-efficacy opioids by studying novel single-molecules with greater MOR selectivity over other opioid receptor types (e.g. kappa-opioid receptor (KOR) or delta-opioid receptor (DOR)) in an assay of locomotor activity in mice as described previously (Santos et al., 2022). The novel opioids studied are shown in **Table 3.1**. listed from high-to-low efficacy at MOR: DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, DC-01-076.1, EG-1-203, and EG-1-230 (Chambers et al., 2022; Gutman et al., 2020, Lutz et al., 2023; *In Press*, Tom Prinsenzano; personal communication)) as well as three reference clinically

available opioids [listed from high-to-low efficacy at MOR]: morphine, buprenorphine, and naltrexone.

When an opioid ligand binds the receptor, a series of secondary signaling pathways are engaged, and this includes the inhibition of the enzyme adenylate cyclase and a reduction in its product cyclic adenosine monophosphate (cAMP), among other effects [**Chapter 1; section 1.2**]. cAMP levels can be reduced to different degrees, and the degree of reduction is dependent on the efficacy of the opioid. In vitro cAMP levels can then be used as a measure of ligand efficacy in this early step of the opioid signaling pathway. Data in **Table 3.1** show maximum levels of in vitro inhibition of forskolin-stimulated adenylate cyclase activity and cAMP accumulation produced by the novel single-molecule opioids in Chinese hamster ovary (CHO) cells that expressed either the human mu-, kappa-, or delta-opioid receptor (Chambers et al., 2022; Gutman et al., 2020, Lutz et al., 2023; *In Press*, Tom Prinsenzano; personal communication). Importantly, the degree of cAMP inhibition can be quantified as the $\%E_{max}$, defined as the maximum effect (E_{max}) of each drug expressed as a percentage of the E_{max} produced by a representative high-efficacy agonist for each receptor type (fentanyl for mu, U50488H for kappa, SNC80 for delta). Additionally, the potency of each drug was quantified as the EC_{50}, defined as the Effective Concentration required to produce 50% of the E_{max} as seen in column 1 of **Table 3.1**. In cases where a drug had a very weak effect to inhibit cAMP accumulation (i.e. a low $\%E_{max}$), then the drug could also be evaluated as an antagonist of the representative high-efficacy agonist at that receptor (fentanyl for mu, U50488H for kappa, SNC80 for delta). In these cases, **Table 3.1.** shows potency expressed as IC_{50} (the Inhibitory Concentration required to produce a 50% decrease in effects of the reference

agonist), and effect magnitude expressed as $\%I_{max}$ (the maximum inhibition of reference-agonist effects).

The $\%E_{max}$ measure shows the maximal degree of cAMP inhibition mediated by each drug at each receptor type, and results can be laid out from highest to lowest $\%E_{max}$ (e.g. DC-01-128.1 has an $\%E_{max}$ of 101 ± 0.2% and EG-1-230 has an $\%E_{max}$ of 33.5 ± 5.9% in cells expressing MOR). Note that E_{max} values in the assay of drug-induced inhibition of forskolin-stimulated cAMP accumulation are higher than E_{max} values in the assay of ligand-stimulated GTPɣS binding as shown in **Table 3.1**. These higher E_{max} values occur because adenylate cyclase inhibition (cAMP inhibition assay) is downstream of G-protein activation (GTPɣS binding assay) in the MOR coupled signaling pathway, and this provides an opportunity for signal amplification. However, the relative rank order of E_{max} values is similar as shown in **Table 3.2.** Receptor selectivity can be observed from the EC_{50} or IC_{50} potency values at MOR vs KOR and DOR. For example, the EC_{50} value for DC-01-128.1 to inhibit cAMP accumulation in cells expressing MOR was 0.07 nM. By contrast, DC-01-128.1 had a 138-fold lower potency at DOR (EC_{50}=9.69 nM) and a 3,396-fold lower potency at KOR, where it functioned only as an antagonist (IC_{50}=2337.7nM). Thus, DC-01-128.1 functioned as a high-efficacy, potent, and highly selective MOR agonist. The other compounds showed graded MOR efficacy, and all were MOR selective. Thus, the goal of the present study was to evaluate the effects of these compounds on opioid-induced locomotor activation in male and female mice. We predicted that the in vitro $\%E_{max}$ as a measure of MOR efficacy to inhibit cAMP will agree with the in vivo E_{max} to induce hyperlocomotor activity in mice.

Table 3.1: In Vitro effects of clinically available and novel single-molecule opioids.

	In Vitro cAMP						
	MOR		KOR		DOR		
Drug name	EC50 ± SEM (nM) (%Emax ± SEM)	IC50 ± SEM (nM) (%Imax ± SEM)	EC50 ± SEM (nM) (%Emax ± SEM)	IC50 ± SEM (nM) (%Imax ± SEM)	EC50 ± SEM (nM) (%Emax ± SEM)	IC50 ± SEM (nM) (%Imax ± SEM)	Reference
Morphine	6.28 ± 0.43 (102.1 ± 0.2%)	N/D	N/D		N/D		Chambers et al 2022
DC-01-128.1	0.07 ± 0.02 (101 ± 0.2%)	N/D	>10,000	237.7 ± 52.6 (114.8 ± 11.2%)	9.69 ± 2.23 (74.5 ± 2.3%)	N/D	Chambers et al 2022
Buprenorphine	0.4 ± 0.36 (100.25 ± 0.67%)	N/D	3.62 ±0.89 (66.27 ± 6.67)	N/D	N/D		Tom Prinsenzano (Personal communication)
DC-01-76.2	1.44 ± 0.48 (94.7 ± 3.1%)	N/D	>10,000	74.0 ± 30.5 (97.1 ± 9.2%)	>10,000	112.9 ± 43.6 (119.1 ± 15.5%)	Chambers et al 2022
EWB-3-14	0.4 ± 0.12 (91%)	N/D	>10,000	54.39 ± 14.28 (93.5 ± 2.3%)	3.2 ± 2.5 (35%)	N/D	Tom Prinsenzano (Personal communication)
JL-02-0039	0.91 ± 0.46 (85.0 ± 5.1%)	N/D	> 10000	111± 45 (82.5 ± 6.5%)	3.07 ± 3.10 (38.5 ± 2.6%)	N/D	Lutz et al 2023 *In Press*
DC-01-0076.1	2.12 ± 0.45 (67.3 ± 6.8%)	45.6 ± 17.3 (18.8 ± 3.8%)	>10,000	19.8 ± 9.7 (101.8 ± 19.1%)	57.0 ± 21.4 (22.6 ± 3.3%)	N/D	Chambers et al 2022
EG-1-203	0.95 ± 0.35 (63.3 ± 3.9%)	N/A	>10,000	N/A	>10,000	N/A	Gutman et al 2020
EG-1-230	2.31 ± 0.78 (33.5 ± 5.9%)	N/A	>10,000	N/A	>10,000	N/A	Gutman et al 2020
Naltrexone	2.14 ± 1.2 (29.6 ± 6.4%)	10.8 ± 1.0 (103.5 ± 0.6%)	0.64 ± 0.32 (56.5 ± 7.2%)	5.53 ± 1.02 (41.3 ± 6.8%)	>10,000	295.1 ± 47.5 (99.4 ± 1.1%)	Chambers et al 2022

Table 3.2: E_{max} values of the cAMP inhibition and GTPɣS binding in MOR.

Drug name	cAMP inhibition %Emax ± SEM	GTPɣS %Emax ± SEM
DC-01-128.1	102.1 ± 0.2	75.4 ± 3.8
DC-01-76.2	101 ± 0.2	29.1 ± 0.8
EWB-3-14	94.7 ± 3.1	20.8 ± 1.7
JL-02-0039	85.0 ± 5.1	13.0 ± 1.5
DC-01-76.1	67.3 ± 6.8	10.5 ± 0.8
EG-1-203	63.3 ± 3.9	4.8 ± 0.6
EG-1-230	33.5 ± 5.9	0.7 ± 0.8

3.2. Methods

3.2.1 Animals

Subjects were male and female ICR mice (Envigo, Frederick, MD) that were 6–8 weeks old upon arrival to the laboratory. Males weighed 27-50 g and females weighed 23–38 g throughout the study. Mice were single-housed in cages with corncob bedding (Envigo), a "nestlet" composed of pressed cotton (Ancare, Bellmore, NY), a cardboard tube for enrichment, and ad libitum access to food (Teklad LM-485 Mouse/Rat Diet; Envigo). Cages were mounted in racks in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed during the light phase of the daily light/dark cycle beginning 1 week after arrival at the laboratory. Ethical animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

3.2.2 Apparatus

Horizontal locomotor activity was assessed as described previously (Santos et al., 2022) during 60-minute sessions in test boxes (16.8 × 12.7 cm2 floor area × 12.7 cm high) housed in sound-attenuating chambers (Med Associates, St. Albans, VT) and located in a procedure room separate from the housing room. Each box had black plexiglass walls, a clear plexiglass ceiling equipped with a house light, bar floors, and six photobeams arranged at 3-cm intervals across the long wall and 1 cm above the floor. Beam breaks were monitored by a microprocessor operating Med Associates software.

3.2.3 Procedure

For all drugs except EG-1-230, a different group of 12 mice (six females, six males) was used to test each drug. One mouse assigned to the EG-1-230 group died before testing began, so this group included 11 mice (six females, five males). Within each group, test sessions were conducted twice a week with at least 48 hours between sessions. All mice received a vehicle control and all doses of the designated test drug, and dose order was randomized across mice using a Latin-square design. The experimenter was not blinded to treatment because data collection was automated by the Med Associates software. There were no exclusion criteria, and all data from all mice were included in final analysis. On test days, mice were brought to the procedure room at least 1 hour before session onset. After subcutaneous (SC) test-drug administration, mice were returned to their home cages for the 5-minute pretreatment interval and then placed into the locomotor activity boxes for a 60-min test session. Doses for each drug were varied in 0.5 or 1.0 log-unit increments across a >10-fold dose range with the intent of progressing from low doses that produced little or no effect to high doses that produced

maximal increases in locomotor activation for that drug. The final dose ranges for each drug were as follows: tianeptine (10-100 mg/kg), DC-01-128.1 (0.1-3.2 mg/kg), DC-01-76.2 (0.1-3.2 mg/kg), EWB-3-14 (0.1-32 mg/kg), JL-02-0039 (1.0-32 mg/kg), DC-01-76.1 (0.32-32 mg/kg), EG-1-203 (3.2-32 mg/kg), and EG-1-230 (3.2-32 mg/kg). For all drugs, antagonism studies were conducted after completion of drug-alone studies in the same mice using one of two experimental designs. First, to determine effectiveness of the antagonist naltrexone to block effects of higher efficacy test compounds, 1.0 mg/kg naltrexone was administered SC 10 min before SC administration of a selected dose of the test drug, and test sessions began 5 min after the test drug. Second, to determine if the effectiveness of lower efficacy test compounds to block locomotor activating effects of morphine, the test drug was administered SC 10 min before 32 mg/kg SC morphine, and test sessions began 5 min after morphine administration.

3.2.4. Data and Statistical Analysis

The primary dependent variable was the total number of beam breaks, excluding consecutive interruptions of the same beam, during each 60-min session. To construct and analyze dose-effect curves for each drug, data were normalized in a two-step process. First, locomotor data in each mouse at each drug dose were expressed as a "Difference Score" relative to vehicle control data in that group using the equation Difference Score = Test – Group Vehicle, where Test equals the locomotor counts in a given mouse after a given drug dose, and Group Vehicle equals the mean locomotor counts after vehicle treatment in that group. Second, the Difference Score in each mouse at each dose was then expressed as a percentage of the mean maximum Difference

Score produced by the reference agonist methadone using the equation % Methadone Emax = (Difference Score / Methadone Emax) * 100.

The resulting dose-effect data were then evaluated in a sequence of steps as we have described previously (Diester et al., 2019; Santos et al., 2022). First, because sex was not the primary variable of interest, pooled data from both females and males were analyzed by repeated-measures one-way ANOVA with dose as the single variable. A significant ANOVA was followed by a Holm-Sidak post-hoc test, and for this and all other parametric statistics, the criterion for significance was $P < 0.05$. Second, pooled dose-effect data were also evaluated to determine E_{max} and ED_{50} values for each drug. The E_{max} was defined as the mean maximum effect (95% confidence limits) produced by any drug dose. The ED_{50} was defined as the dose producing 50% of the E_{max} value for that drug, and ED_{50} values (95% confidence limits) were determined by linear regression of the linear ascending portion of the dose-effect curve. E_{max} and ED_{50} values were considered to be significantly different across drugs if 95% confidence limits did not overlap. Lastly, to provide preliminary information regarding potential sex differences in drug effects, data for each drug were segregated by sex and compared by two-way ANOVA with sex as a between-subjects factor and drug dose as a within-subjects factor. A significant sex × dose interaction was followed by a Holm-Sidak post-hoc test. Additionally, the two-way ANOVA results were submitted to post hoc power analyses to calculate the Cohen's f effect size, achieved power ($1 - \beta$), and the total number of animals predicted as necessary to achieve power ≥ 0.8. For antagonism experiments, raw data were analyzed as appropriate by t-test or by one-way ANOVA followed by a Dunnett's post hoc test. Power analysis was conducted using the free statistical analysis program

G*Power (Faul et al., 2007), and all other analyses were conducted using GraphPad Prism 9.5 (La Jolla, CA).

3.2.5. Drugs

(±) Methadone HCl and naltrexone HCl were provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Tianeptine sodium salt was purchased from Cayman Chemical (Ann Arbor, MI). DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, DC-01-76.1, EG-1-203 HBr, and EG-1-230 HBr were provided by the Drug Design and Synthesis Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism (Bethesda, MD). For in vivo studies of locomotor activity, methadone, naltrexone, tianeptine, and EG-1-230 were dissolved in sterile saline, and all other compounds were dissolved in a 1:1:18 vehicle consisting of 5% ethanol, 5% emulphor, and 90% saline. Doses were calculated using the salt or free-base form of each drug described above and were administered subcutaneously (SC) in a volume of 10 ml/kg.

3.3. Results

Locomotor Activity

Table 3.3 shows the mean ± SEM number of baseline locomotor counts after vehicle administration in each group of mice. There was a significant difference in baseline activity across groups [F (8,98) =7.438, p<0.0001], and follow-up analysis by two-way ANOVA to include sex as a variable confirmed a main effect of group [F(8,89)=7.394), p<0.0001] but no main effect of sex (p=0.195) and no group x sex interaction (p=0.560). To control for the different levels of baseline activity in each group,

raw data for each drug were transformed to Difference Scores and expressed as a percentage of the mean E_{max} Difference Score produced by the reference drug methadone. **Figure 3.1** uses the data with methadone to illustrate the sequence of data analysis steps that was followed for each drug as described in Methods. Methadone produced a dose-dependent increase in locomotor activity. **Table 3.3** shows the one-way ANOVA results, E_{max} and ED_{50} for methadone pooled across sexes, and **Table 3.4** shows the two-way ANOVA results and post hoc power analysis for methadone segregated by sex. There was no main effect of sex or sex x dose interaction for methadone.

Table 3.3: Baseline activity and results of dose-effect analyses for locomotor activating effects of opioids in each group of female and male ICR mice.

Drugs	Baseline ± SEM # Counts	Emax (95% CI) % Methadone E_{max}	ED50 (95% CL) mg/kg	One-way ANOVA
Methadone	2350.3 ± 264.7	100 (82.8-117.2)	2.50 (1.61-3.99)	F (2.898, 31.87) = 38.80; P<0.0001
Tianeptine	1248.6 ± 127.6	102.8 (84.4-123.2)	18.28 (16.37-20.46)	F (1.884, 20.73) = 86.74; P<0.0001
DC-01-128.1	2692.3 ± 241.2	80.1 (68.9-91.2)	0.19 (0.10-0.29)	F (2.946, 32.40) = 31.22; P<0.0001
DC-01-76.2	2732.3 ± 706.9	87.6 (50.0-125.3)	0.47 (0.16-0.80)	F (1.942, 21.36) = 19.59; P<0.0001
EWB-3-14	1570.5 ± 51.2	101.5 (67.6-135.4)	1.05 (0.77-1.44)	F (2.824, 31.06) = 22.36; P<0.0001
JL-02-0039	3102.8 ± 151.9	87.1 (66.6-107.7)	2.85 (1.31-4.81)	F (2.175, 23.93) = 40.55; P<0.0001
DC-01-0076.1	3072.6 ± 156.4	34.3 (16.6-52.0)	0.72 (0.47-1.74)	F (2.283, 25.12) = 7.740; P=0.0017
EG-1-203	989.3 ± 125.2	13.0 (2.5-23.5)	5.42 (Not Determined)	F (1.754, 19.29) = 5.112; P=0.0196
EG-1-230	1338.8 ± 261.4	2.2 (-5.8-10.1)	Inactive	F (2.434, 24.34) = 0.2863; P=0.7943

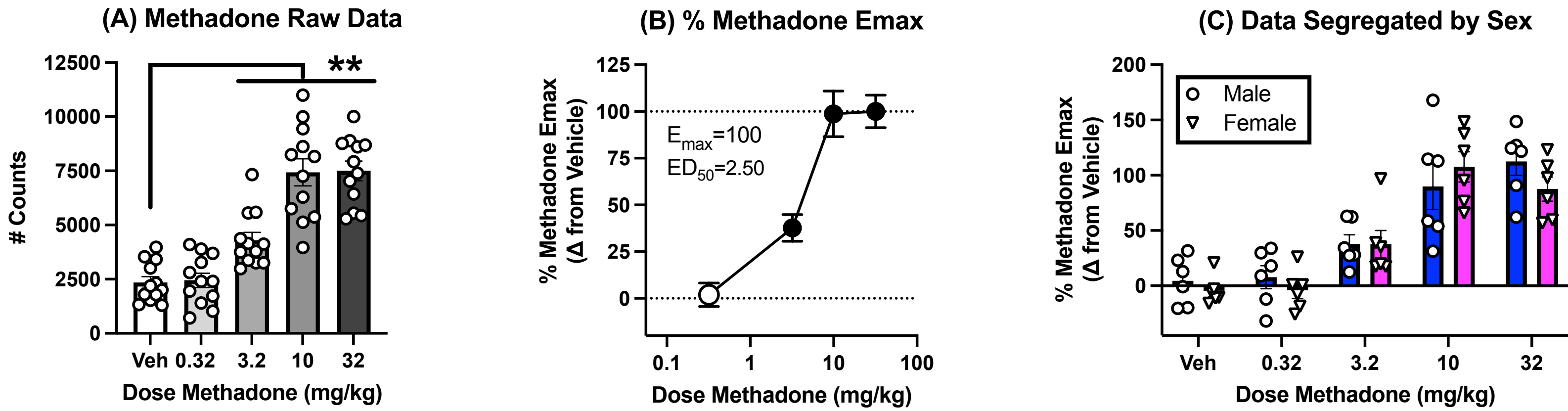


Figure 3.1: Experimental design and analysis illustrated with methadone. Each drug was tested in a separate group of 11-12 mice (6 females, 5-6 males) using a within-subjects repeated-measures design. (A) Initial analysis pooled raw data from both sexes. Abscissa: dose methadone in mg/kg administered SC. Veh=vehicle. Ordinate: Total locomotor activity counts during a 60 min test session. Bars show mean±SEM, and points show individual data. ** Asterisks indicate different from vehicle as indicated by one-way ANOVA followed by a Holm-Sidak post hoc test, p<0.01. (B) For calculation of dose-effect parameters (Emax, ED50), data for each mouse at each dose were transformed to % Methadone Emax using the equation [(Drug - Veh)/(5149.8)]*100, where Drug = total locomotor counts in a given mouse after a drug dose, Veh = mean locomotor counts after vehicle in that group, and 5149.8 = the mean maximum increase in locomotor counts produced by 32 mg/kg methadone in the methadone group. Filled symbols indicate different from vehicle as indicated by one-way ANOVA followed by a Holm-Sidak post hoc test, p<0.05. (C) Data in panel B were segregated by sex and analyzed by two-way ANOVA. In this case, there was a main effect of dose [F(2.82, 28.2)=38.74, p<0.0001], but no main effect of sex [F1,10)=0.42, p=0.53] and no sex x dose interaction [F(4,40)=0.98, p=0.43].

Figure 3.2 shows dose-effect curves for data pooled across sexes for each test drug. One-way ANOVA, E_{max}, and ED_{50} values are shown in **Table 3.3.** All drugs except EG-1-230 produced dose-dependent and significant increases in locomotor activity. E_{max} values were statistically similar (as indicated by overlapping 95% confidence limits) for the reference agonist methadone and the test compounds tianeptine, DC-01-128.1, DC-01-76.2, EWB-3-14, and JL-02-0039. Conversely, the test compounds DC-01-76.1, EG-1-203, and EG-1-230 had lower E_{max} values than methadone and the other test compounds (except for an overlap in E_{max} 95% confidence limits for DC-01-76.2 ≥ DEC-01-76.1). Lastly, the E_{max} for EG-1-230 was also lower than that for DC-01-76.1. The potency rank order of all compounds as determined by ED_{50} values was DC-01-128.1 > DC-01-76.2 ≥ DC-01-76.1 ≥ EWB-3-14 > methadone ≥ JL-02-0039 > EG-1-203 > tianeptine. An ED_{50} value for EG-1-230 could not be determined because it was inactive when administered alone. Two-way ANOVA results for data segregated by sex for each drug are shown in **Table 3.4.** For most groups, there was not a significant main effect of sex or sex x dose interaction. As the only exception, there was a significant sex x dose interaction for EG-1-203 [F (3, 30) = 3.77; P=0.0208]; however, post-hoc analysis did not indicate a significant effect of sex at any dose.

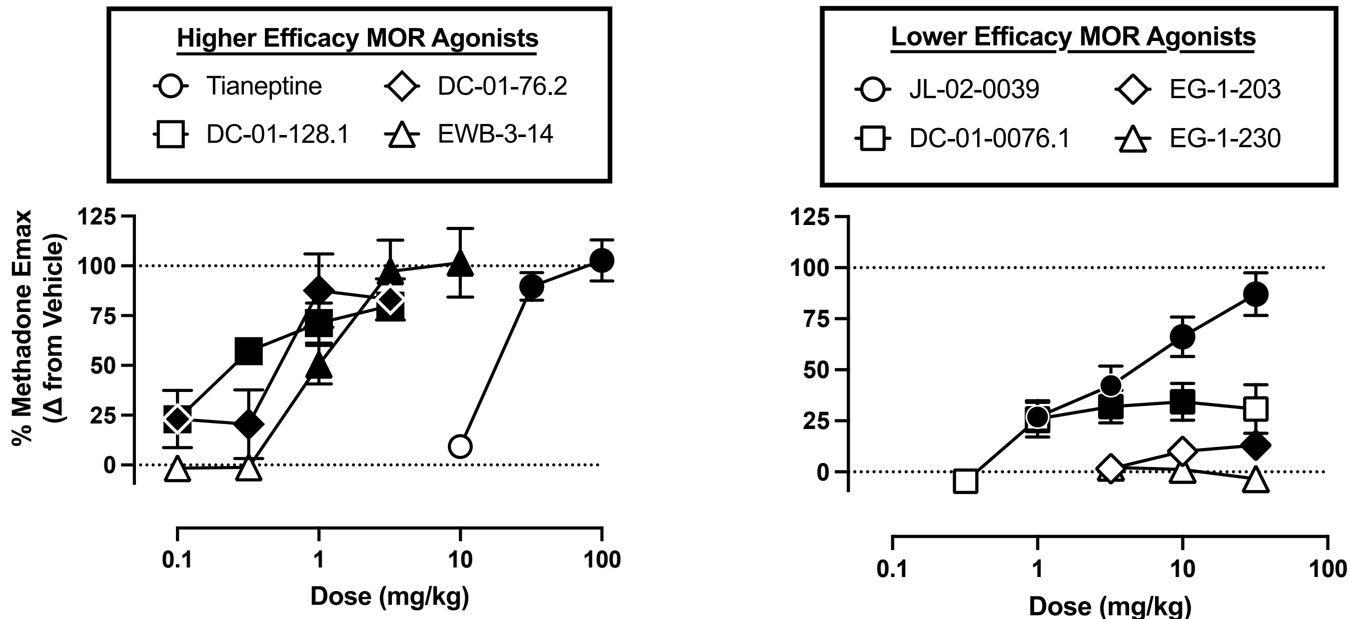


Figure 3.2: Locomotor activating effects of opioids in male and female ICR mice. Abscissae: dose in mg/kg administered SC (log scale). Ordinates: Locomotor activating effects expressed as a percent of the methadone Emax. Points show mean ± SEM, and filled points indicate doses that produced effects significantly greater than Veh ($p<0.05$).

Table 3.4: Two-way ANOVA results and post hoc power analyses to assess the role of sex as a determinant of opioid-induced locomotor activating in female and male ICR mice.

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Methadone	Dose Main Effect	0.795	F (2.82, 28.20) = 38.74; P<0.0001	1.968	1	3
	Sex Main Effect	0.015	F (1, 10) = 0.42; P=0.5335	0.125	0.123	>100
	Dose x Sex Interaction	0.089	F (4, 40) = 0.98; P=0.4286	0.313	0.739	14
Tianeptine	Dose Main Effect	0.888	F (1.89, 18.85) = 79.10; P<0.0001	2.813	1	3
	Sex Main Effect	0.00007	F (1, 10) = 0.001; P=0.9745	0.009	0.050	>100
	Dose x Sex Interaction	0.003	F (3, 30) = 0.03; P=0.9924	0.056	0.066	>100
DC-01-128.1	Dose Main Effect	0.766	F (2.58, 25.83) = 32.75; P<0.0001	1.809	1	3
	Sex Main Effect	0.061	F (1, 10) = 1.45; P=0.2560	0.254	0.357	34
	Dose x Sex Interaction	0.133	F (4, 40) = 1.54; P=0.2090	0.392	0.919	10
DC-01-76.2	Dose Main Effect	0.683	F (1.90, 19.04) = 21.55; P<0.0001	1.468	1	4
	Sex Main Effect	0.0003	F (1, 10) = 0.001; P=0.9743	0.018	0.052	>100
	Dose x Sex Interaction	0.173	F (4, 40) = 2.09; P=0.0992	0.458	0.979	8
EWB-3-14	Dose Main Effect	0.678	F (2.74, 27.36) = 21.08; P<0.0001	1.452	1	4
	Sex Main Effect	0.004	F (1, 10) = 0.05; P=0.8227	0.059	0.066	>100
	Dose x Sex Interaction	0.0357	F (6, 60) = 0.37; P=0.8952	0.192	0.377	28
JL-02-0039	Dose Main Effect	0.796	F (2.11, 21.07) = 38.95; P<0.0001	1.97	1	3
	Sex Main Effect	0.010	F (1, 10) = 0.05; P=0.8336	0.103	0.099	>100
	Dose x Sex Interaction	0.054	F (4, 40) = 0.57; P=0.6885	0.238	0.474	23
DC-01-0076.1	Dose Main Effect	0.448	F (2.33, 23.28) = 8.11; P=0.0014	0.900	0.999	5
	Sex Main Effect	0.217	F (1, 10) = 2.93; P=0.1179	0.527	0.907	10
	Dose x Sex Interaction	0.132	F (5, 50) = 1.52; P=0.1991	0.390	0.949	9
EG-1-203	Dose Main Effect	0.390	F (1.75, 17.54) = 6.39; P=0.0101	0.800	0.999	6
	Sex Main Effect	0.456	F (1, 10) = 2.30; P=0.1601	0.915	0.999	6
	Dose x Sex Interaction	0.274	F (3, 30) = 3.77; **P=0.0208**	0.614	0.999	6
EG-1-230	Dose Main Effect	0.031	F (2.43, 21.90) = 0.29; P=0.7949	0.178	0.204	53
	Sex Main Effect	0.012	F (1, 9) = 0.30; P=0.5953	0.108	0.099	>100
	Dose x Sex Interaction	0.012	F (3, 27) = 0.11; P=0.9553	0.109	0.109	>100

Figure 3.3 compares the time courses of vehicle and the E_{max} drug dose in each group during the 60-minute session. Vehicle treated animals had high initial locomotor activity followed by a decline to lower levels later in the session. Drug-induced increases in locomotor activity were generally observed within the first 10-15 min of the session and were sustained for the duration of the session.

Figure 3.4 shows the results of antagonism studies to determine receptor mechanisms of drug action. Naltrexone significantly attenuated the effects of locomotor-activating doses of tianeptine, DC-01-128.1, DC-01-76.2, EWB-3-14, JL-02-0039, and DC-01-76.1. Conversely, the lower efficacy compounds EG-1-203 and EG-1-230 both significantly attenuated the locomotor-activating effects of morphine.

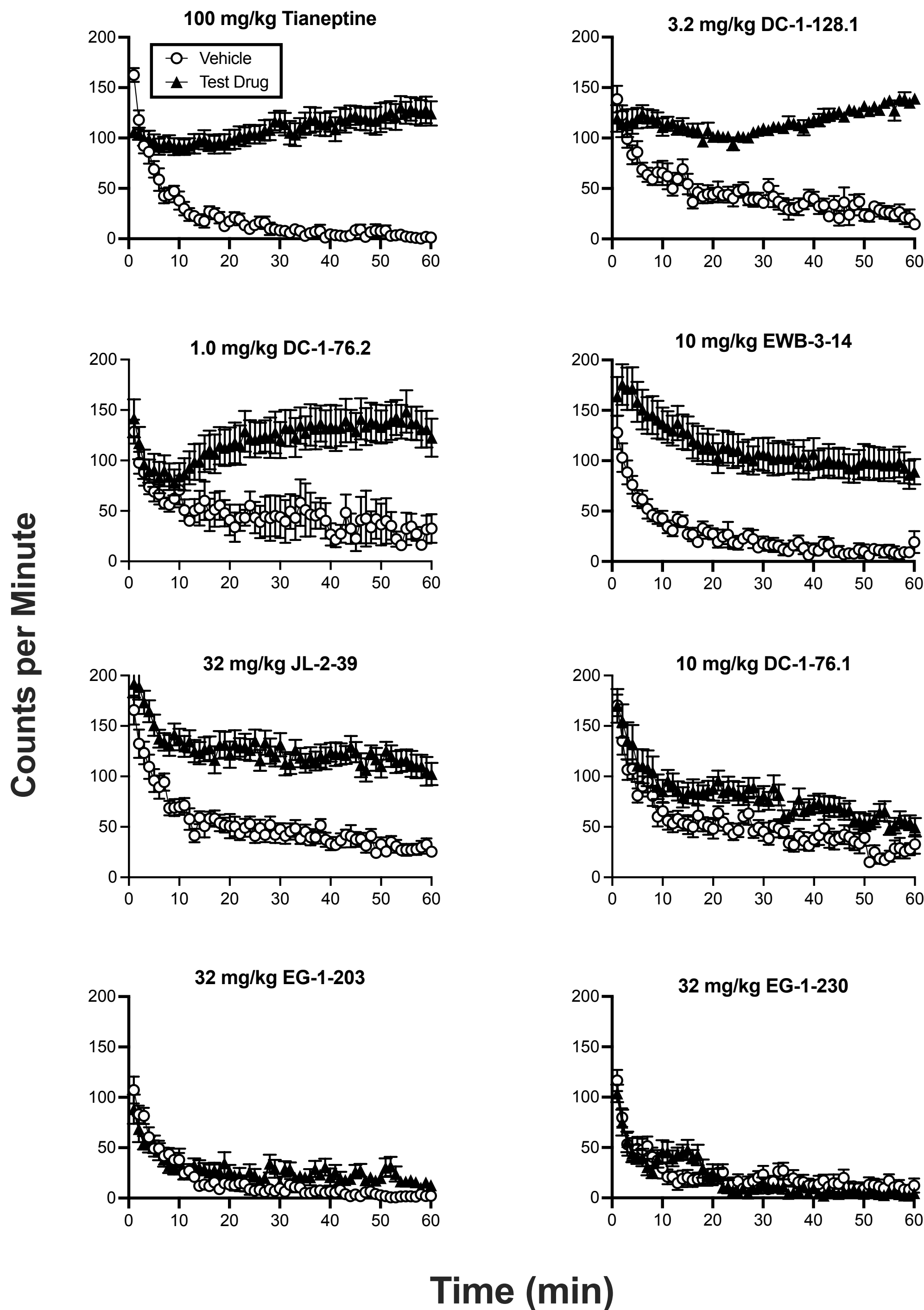
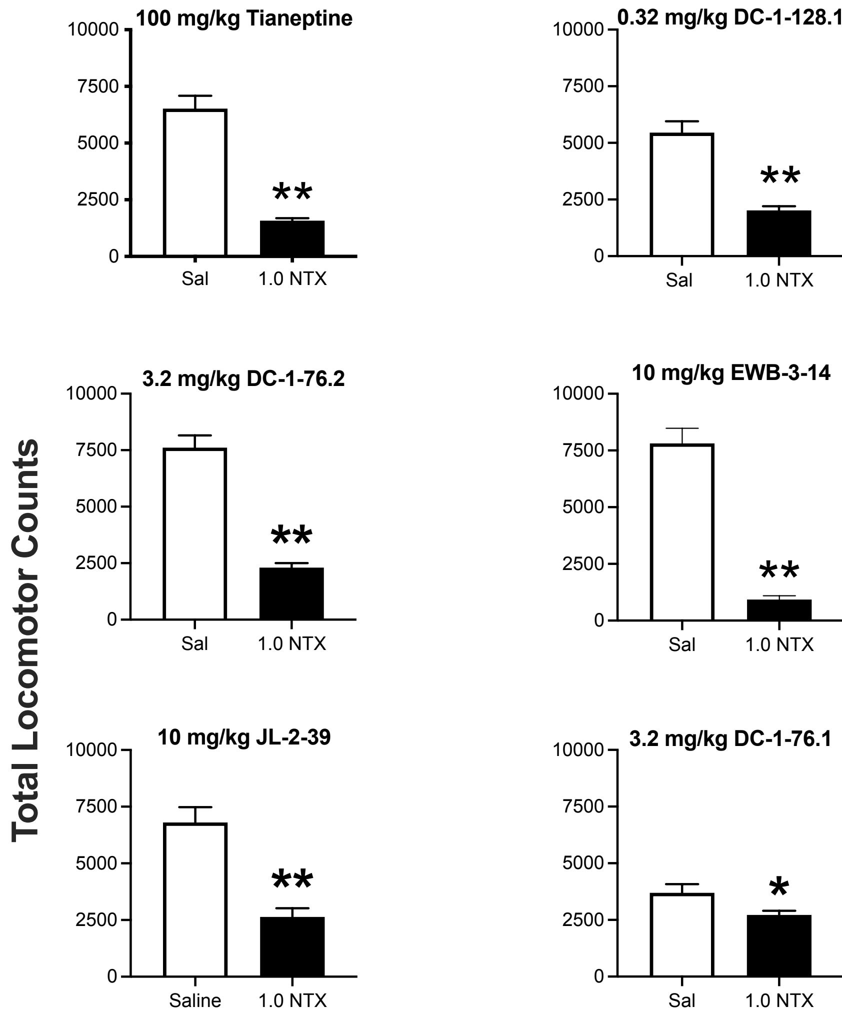


Figure 3.3: Time course of Emax dose for each drug. Abscissae: Time in minutes of test session, which began 5 min after SC drug administration. Ordinates: Number of locomotor counts per minute. All points show mean ± SEM from 11-12 mice.



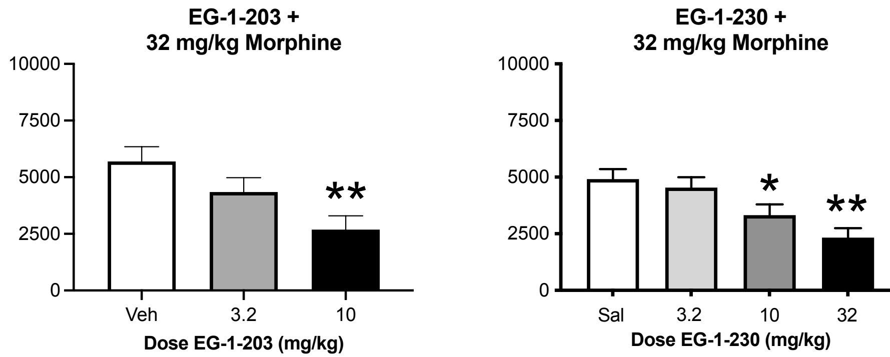


Figure 3.4: Receptor mechanisms of test-drug effects

Figure 3.4: Receptor mechanisms of test-drug effects [figure legend]. Abscissae: Treatment. Ordinates. Number of locomotor counts during a 60-min session. For the top 6 panels, the test drug was administered after pretreatment with either saline (Sal) or 1.0 mg/kg naltrexone (1.0 NTX) to evaluate sensitivity of test drug-induced locomotor stimulation to naltrexone antagonism. For each test drug, the dose tested is indicated in the panel header and was the second or third lowest dose to produce significant locomotor stimulation. For the bottom 2 panels, the test drugs produced little or no locomotor stimulation on their own. Accordingly, they were evaluated for their effectiveness to antagonize locomotor activating effects of 32 mg/kg morphine. All bars show mean ± SEM for 11-12 mice. Asterisks indicate significant antagonism, * $p<0.05$, ** $p<0.01$.

3.4. Summary

This study evaluated locomotor activity in male and female mice by a series of novel single-molecule opioids that are selective and vary in their efficacy at MOR. There were three main findings. First, the results presented here support previous findings for the efficacy-dependent MOR agonist effects to produce maximal locomotor activation in mice. Second, the drug induced and sustained time course effects of the novel single-molecule opioids were efficacy dependent. Finally, these novel single-molecule opioids showed mu-receptor mechanism of action as determined by a series of antagonism studies. Findings here support the in vitro efficacy requirement to inhibit cAMP and to produce in vivo maximal locomotor stimulation in mice.

Chapter Four

Climbing Behavior by Mice as an Endpoint for Preclinical Assessment of Drug Effects in the Absence and Presence of Pain

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4.1. Introduction

Clinically relevant pain is often associated with impaired function and behavioral depression, and a common goal of pain treatment is to alleviate these manifestations of pain and restore normal behavior (Cleeland and Ryan, 1994; Dworkin et al., 2005). Preclinical research using experimental pain models can focus on parallel endpoints of "pain-depressed behavior," which can be defined as behaviors that decrease in rate, frequency, or intensity after delivery of a noxious stimulus (Negus et al., 2010a; Negus, 2019). There are two main advantages to studying pain-depressed behaviors as a category of endpoints for research on expression, mechanisms, and treatment of pain. First, preclinical-to-clinical translational research in any domain is optimized when preclinical studies measure endpoints homologous to clinically relevant human endpoints (González-Cano et al., 2020; Yu, 2011), and as noted above, preclinical endpoints of pain-depressed behavior are homologous to clinical endpoints of pain-related functional impairment and behavioral depression (Cobos et al., 2012). Second, pain-depressed behaviors are not susceptible to false positive effects observed with drugs that cause motor impairment because effective analgesics will increase the expression of the pain-depressed behaviors, whereas drugs that produce motor impairment only exacerbate pain-related behavioral depression (Negus, 2019, 2013).

In an effort to develop valid and efficient assays of pain-depressed behavior, experimental models of acute and chronic pain have been evaluated for their effectiveness in rodents to decrease a range of different behaviors, including horizontal locomotion (Hasriadi et al., 2021; Stevenson et al., 2009), wheel running (Cobos et al., 2012), and nesting (Diester et al., 2021; Jirkof, 2014) by mice. The present study sought to evaluate pain-related depression of another behavior: climbing. Climbing is an ethologically important component of locomotor behavior in rodents (Innes et al., 2018; Makowska and Weary, 2016), and it consists of vertical locomotion required to navigate vertically oriented surfaces in the wild. However, climbing is rarely examined in laboratory environments, where home cages and behavioral testing chambers are usually shallow and have smooth walls that cannot be scaled. Other types of test environments with taller profiles and scalable vertical surfaces have occasionally been used to assess climbing in mice (Marcais-Collado et al., 1983; Urban et al., 2011), but these types of environments have not yet been used to assess the effects of experimental pain models in the absence or presence of known or candidate analgesics.

Accordingly, the goal of this study was to use a vertically oriented cylinder with wire-mesh walls as a test environment to assess the expression and treatment of pain-related depression of climbing in mice. Initial validation of the procedure proceeded in four steps. First, we evaluated the expression and stability of climbing during repeated, within-subject testing to assess suitability of climbing for a within-subjects experimental design. Second, we determined the effectiveness of intraperitoneal injection of dilute lactic acid (IP acid) as an acute noxious stimulus to decrease climbing. IP acid injection models tissue acidosis associated with many types of pain states (Reeh and Steen,

1996), and we have shown previously that it produces a concentration-dependent depression of a wide range of different behaviors in mice and rats (Baldwin et al., 2022; Negus, 2013; Negus et al., 2015). Third, we evaluated the effects of the positive-control non-steroidal anti-inflammatory drug (NSAID) ketoprofen to block IP acid-depressed climbing. Ketoprofen is a clinically effective analgesic, and we have previously shown that it blocks IP acid-induced depression of a range of different behaviors in both mice and rats (Diester et al., 2021; Negus et al., 2015; Stevenson et al., 2009, 2006). Finally, we evaluated the effects of the centrally acting kappa opioid receptor (KOR) agonist U69593 as a negative control. Centrally acting KOR agonists represent one class of candidate analgesics that has produced analgesia-like effects in conventional preclinical procedures but that has failed to produce reliable and safe analgesia in humans (e.g. (Pande et al., 1996)) and similarly fails to alleviate pain-related behavioral depression preclinically (Lazenka, 2021; Negus et al., 2015, 2010b; Wilkerson et al., 2018).

Following the initial validation process, we investigated the role of mu-opioid receptor (MOR) ligand efficacy as a determinant of MOR agonist effectiveness to block IP acid-induced depression of climbing. High-efficacy MOR agonists like fentanyl and morphine are clinically effective analgesics, but their use is limited by side effects such as respiratory depression, impaired motor function, inhibition of gastrointestinal transit, tolerance, dependence, and abuse liability (Hong et al., 2008; Nafziger and Barkin, 2018; “Prescription Opioids | Drug Overdose | CDC Injury Center,” 2019). Lower efficacy MOR agonists like buprenorphine retain clinically effective analgesic effects, but they produce fewer and weaker side effects and are therefore safer (Davis, 2012; Ehrlich and Darcq, 2019; White et al., 2018), but are rarely used (Dowell et al., 2022). As a result, the

development of novel, selective, low-efficacy MOR agonists may represent a promising path for analgesic drug development (Altarifi et al., 2015b, 2015a; Chakraborty et al., 2021). MOR efficacy may be especially relevant for opioid effects in assays of pain-depressed behavior, where opioids can produce competing effects that include both analgesia (which alleviates pain-related behavioral depression and increases rates of the target behavior) and motor impairment (which can reduce rates of the target behavior and obscure analgesic restoration of pain-depressed behavior) (Altarifi et al., 2015a; Baldwin et al., 2022; Garner et al., 2021). Accordingly, we manipulated MOR efficacy by testing both (a) a set of single-molecule opioids with decreasing MOR efficacy (fentanyl > buprenorphine > naltrexone) (Altarifi et al., 2015a; Santos et al., 2022; Selley et al., 1998), and (b) a series of fixed-proportion fentanyl/naltrexone mixtures that vary in net MOR efficacy as described previously (Cornelissen et al., 2018; Santos et al., 2022; Schwienteck et al., 2019; Selley et al., 2021). We hypothesized that low-efficacy single-molecule opioids or fentanyl/naltrexone mixtures would have sufficient efficacy to alleviate pain-related depression of climbing without affecting motor behavior.

4.2. Methods and materials

4.2.1 Subjects.

Subjects were male and female ICR mice (Envigo, Frederick, MD) that were 6–8 weeks old upon arrival to the laboratory. ICR mice were used in this study because it is an outbred strain of mice and outbred strain of mice have been recommended as being advantageous in pain studies (Tuttle et al., 2018). Males weighed 27-50 g and females weighed 23–38 g throughout the study. Mice were generally housed in same-sex, littermate groups of three mice per cage with corncob bedding (Envigo), a “nestlet”

composed of pressed cotton (Ancare, Bellmore, NY), a cardboard tube for enrichment, and ad libitum access to food (Teklad LM-485 Mouse/Rat Diet; Envigo). In some cases, males were split into smaller groups or isolated to minimize fighting. Cages were mounted in a RAIR HD Ventilated Rack (Laboratory Products, Seaford, DE) in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed during the light phase of the daily light/dark cycle beginning 1 week after arrival at the laboratory. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

4.2.2. Apparatus and Climbing Assessment.

To assess climbing, mice were transported to an experimental room separate from the housing room and placed individually into clear plastic cylinders (11.25 cm diameter x 25.5 cm tall; see Figure 1) for 10-min behavioral sessions. Each cylinder was lined from bottom to top with 0.5 cm^2 aluminum wire mesh around 75% of the inner perimeter (26.03 cm width x 24.13 cm height; no mesh in front to permit unobscured video recording). Additionally, the top of the cylinder was covered by a lid made from the same wire mesh. During each session, three mice were tested at once in separate cylinders. Cardboard barriers between the cylinders prevented visual contact between mice during testing, and behavior was recorded with a video camera (Amazon, Inc GordVE Video Camera Camcorder HD 1080P) or an iPad (Apple Inc, 2011) with the experimenter absent from the room. The main dependent variable was the amount of time mice spent climbing

during each 10-min behavioral session. “Climbing” was defined as any time that a mouse had at least one paw in contact with the mesh wall or lid and all paws off the floor. Time climbing was scored by at least one of two trained observers blind to experimental treatments. A subset of videos was scored by both observers at the beginning of the study and periodically during the study to monitor inter-rater reliability.

4.2.3. Experimental Design and Procedure.

Studies proceeded in two phases to (1) validate the procedure, and (2) test the effects of mu opioid receptor (MOR)-ligand treatments designed to vary the efficacy of MOR activation. Each experiment was conducted using a within-subjects repeated-measures design. Treatments within each group were randomized across subjects using a Latin-square design, and tests were separated by three to four days to permit drug washout between tests.

Initial validation studies proceeded in four steps. Step 1 evaluated the stability of climbing during repeated testing. Mice in this group received no injections and were tested a total of five times at intervals of three to four days to mimic the testing intervals planned for subsequent treatment studies. Step 2 evaluated pain-related depression of climbing produced by intraperitoneal injection of dilute lactic acid (IP acid) as an acute visceral noxious stimulus. Mice in this group were tested with IP water or a range of IP acid concentrations (0.18-0.56%) administered 10 min before each behavioral session. Step 3 evaluated effects of the clinically effective positive-control analgesic and non-steroidal anti-inflammatory drug ketoprofen (10 mg/kg) administered subcutaneously (SC) as a pretreatment to 0.32% IP acid. Mice in this group received four treatments: SC ketoprofen

+ IP acid, SC ketoprofen + IP water, SC saline + IP acid, or SC saline + IP water. SC ketoprofen or its vehicle was administered 30 min before the session, and IP acid or water was administered 10 min before the session. Step 4 evaluated effects of the negative-control kappa opioid receptor (KOR) agonist U69593 administered SC alone or as a pretreatment to 0.32% IP acid. One group of mice was used to evaluate effects of U69593 administered alone (vehicle and 0.1-1.0 mg/kg), and a second group of mice received U69593 (vehicle and 0.1-1.0 mg/kg SC) administered as a pretreatment before 0.32% IP acid. U69593 or its vehicle was administered 20 min before the session, and IP acid was administered 10 min before the session.

Studies to evaluate the effects of MOR activation proceeded in two steps. In Step 1, effects were determined for SC administration of the high-efficacy MOR agonist fentanyl (0.0032-0.1 mg/kg), the intermediate-efficacy MOR agonist buprenorphine (0.01-0.32 mg/kg), and the MOR antagonist naltrexone (0.01-0.1 mg/kg) and their saline vehicles. Each MOR ligand was evaluated both alone in one group of mice and as a pretreatment to 0.32% IP acid in a second group of mice. Step 2 evaluated effects produced by a series of fixed-proportion fentanyl/naltrexone mixtures. We have reported previously that the proportion of fentanyl in fentanyl/naltrexone mixtures can be manipulated such that decreasing fentanyl proportions result in decreasing net efficacy of the mixture (Cornelissen et al., 2018; Santos et al., 2022; Schwienteck et al., 2019; Selley et al., 2021). Here, we examined 10:1, 3.2:1, and 1:1 mixtures of fentanyl/naltrexone. As with the single-molecule MOR ligands, each fentanyl/naltrexone mixture was evaluated both alone in one group of mice and as a pretreatment to 0.32% IP acid in a second group of mice. For all fentanyl/naltrexone mixture studies, the fentanyl

doses were 0.0032-0.1 mg/kg, and the naltrexone doses varied according to the designated proportion. For all MOR ligands and mixtures, the opioid or its vehicle was administered 20 min before the session, and IP acid was administered 10 min before the session.

Mice were randomly assigned to treatment groups, and testing progressed until 12 mice (6 male, 6 female) met inclusion criteria for a given treatment. The only exception was the 10:1 fentanyl/naltrexone + IP acid group, which has data from 11 mice (6 male, 5 female). There were two inclusion criteria. First, all treatment groups included a vehicle control, and mice were included only if they climbed for ≥60 sec under these control conditions. Second, for groups to examine test drug effects as pretreatments to IP acid, mice were included only if drug vehicle + IP acid produced ≥ 20% decrease in climbing time relative to vehicle treatment. The number of mice assigned to each group but failing to meet the inclusion criteria is reported for each group in **Table 4.2.**

4.2.4. Data Analysis.

Behavioral sessions were videotaped and scored by trained observers blinded to experimental treatments. Raw data as “Time Climbing” in sec are reported for the first experiment to examine stability of climbing across days in mice that received no other treatment. For all subsequent analyses with IP acid and test drugs, data in each mouse were transformed to a % of the mean vehicle control data for that mouse’s group using the equation (time climbing after a given treatment in a given mouse ÷ mean time climbing after vehicle control for that mouse’s group) x 100. Raw data (for the first experiment) and transformed data (for all drug ± IP acid experiments) were analyzed in a series of three

steps as described by us previously for studies that include both females and males but are not intended a priori to detect sex differences (Diester et al., 2019). First, because sex was not the primary variable of interest, pooled data from both females and males were analyzed by repeated-measures one-way ANOVA with time or dose as the single variable, and a significant ANOVA was followed by a Dunnett's or Tukey's post hoc test. Second, data were segregated by sex and analyzed by two-way ANOVA with sex as a between-subjects factor and time or dose as a within-subjects factor. A significant main effect of sex or sex × dose interaction was followed by a Holm-Sidak post-hoc test. These first two steps of data analysis were performed using GraphPad Prism 9.0 (La Jolla, CA). Lastly, the two-way ANOVA results were submitted to power analyses to calculate the Cohen's f effect size, achieved power (1 - β), and the total number of animals predicted as necessary to achieve power ≥ 0.8 using the free statistical analysis program G*Power (Faul et al., 2007).

In addition to these within-group analyses, three types of between-group analyses were conducted. First, raw vehicle control data were compared by one-way ANOVA across groups receiving the three different types of vehicle control treatment: SC saline alone, IP water alone, or SC saline + IP water. A significant ANOVA was followed by a Holm-Sidak post hoc test to compare each group to all other groups. Second, raw vehicle control data were also compared by one-way ANOVA across individual groups for which the vehicle control was either SC saline alone or IP water administered alone or in conjunction with SC saline. A significant ANOVA was again followed by a Holm-Sidak post hoc test. Lastly, data from experiments with fentanyl/naltrexone mixtures administered alone were used to determine the efficacy requirement for opioid effects on

climbing as we have described previously (Cornelissen et al., 2018; Santos et al., 2022; Schwienteck et al., 2019; Selley et al., 2021). Briefly, data from each mixture were transformed to a percent of the maximum effect produced by fentanyl alone using the equation [(vehicle - mixture) ÷ (vehicle – fentanyl)] x 100, where "vehicle" equals the mean vehicle control data in a group, "mixture" equals the time climbing in a given mouse after a given dose of a mixture, and "fentanyl" equals the mean maximum effect of fentanyl alone in the fentanyl treatment group. The maximum effect of each mixture was then plotted as a function of the proportion of fentanyl in the mixture, and linear regression was used to determine the EP50 value (95% confidence limits), with EP50 defined as the "effective proportion" of fentanyl to naltrexone required to produce 50% of the maximum fentanyl-alone effect. The EP50 serves as a metric of the efficacy requirement for a given effect, and the EP50 for fentanyl/naltrexone-mixture effects in this study was compared to EP50 values determined in previous studies for fentanyl/naltrexone-mixture effects on other previously reported in vivo and in vitro endpoints. EP50 values were considered to be significantly different if 95% confidence limits did not overlap.

Two observers were trained to score all videos and most videos were scored by only one of these two observers; however, a subset of videos at the beginning of the study and periodically during the study were scored by both observers. Inter-rater reliability was assessed by determining the Pearson's r and P-value for the correlation in observer scores.

4.2.5. Drugs.

Fentanyl HCl, naltrexone HCl, buprenorphine HCl, and U69593 were provided by the National Institute on Drug Abuse Drug Supply Program and were dissolved in sterile saline. Ketoprofen (100 mg/mL; Ford Dodge, IA) was diluted in sterile saline. All drugs were administered subcutaneously (SC) in volumes of 10 ml/kg. Lactic acid was purchased from Sigma-Aldrich (St. Louis, MO), diluted in sterile water, and administered intraperitoneally (IP) in a volume of 10 ml/kg.

4.3. Results

Figure 4.1 shows that climbing in the absence of any treatment was stable with repeated testing, and this figure also illustrates the statistical analysis pipeline for all subsequent experiments. First, data from both sexes were pooled and analyzed by repeated-measures one-way ANOVA (**Figure 4.1.B**). This analysis indicated no effect of test day [$F(2.766, 30.42) = 0.2100$; $P=0.8748$]. Second, data were segregated by sex and analyzed by two-way ANOVA (**Figure 4.1.C**). This analysis indicated no main effect of either Day [$F(2.330, 23.30) = 0.2168$; $P=0.8378$] or Sex [$F(1, 10) = 0.9937$; $P= 0.3423$], and no Day x Sex interaction [$F(4, 40) = 1.355$; $P=0.2668$]. Lastly, the segregated data were submitted to post hoc power analysis and results are shown in **Table 4.1.** In addition, the videos for these experiments were scored by both observers, and results were compared to assess inter-rater reliability (**Figure 4.1.D**). Results from the two observers were significantly correlated (Pearson's $r = 0.9916$; $P<0.0001$). Later checks on inter-rater reliability yielded similarly high Pearson's r values and significant P-values (data not shown).

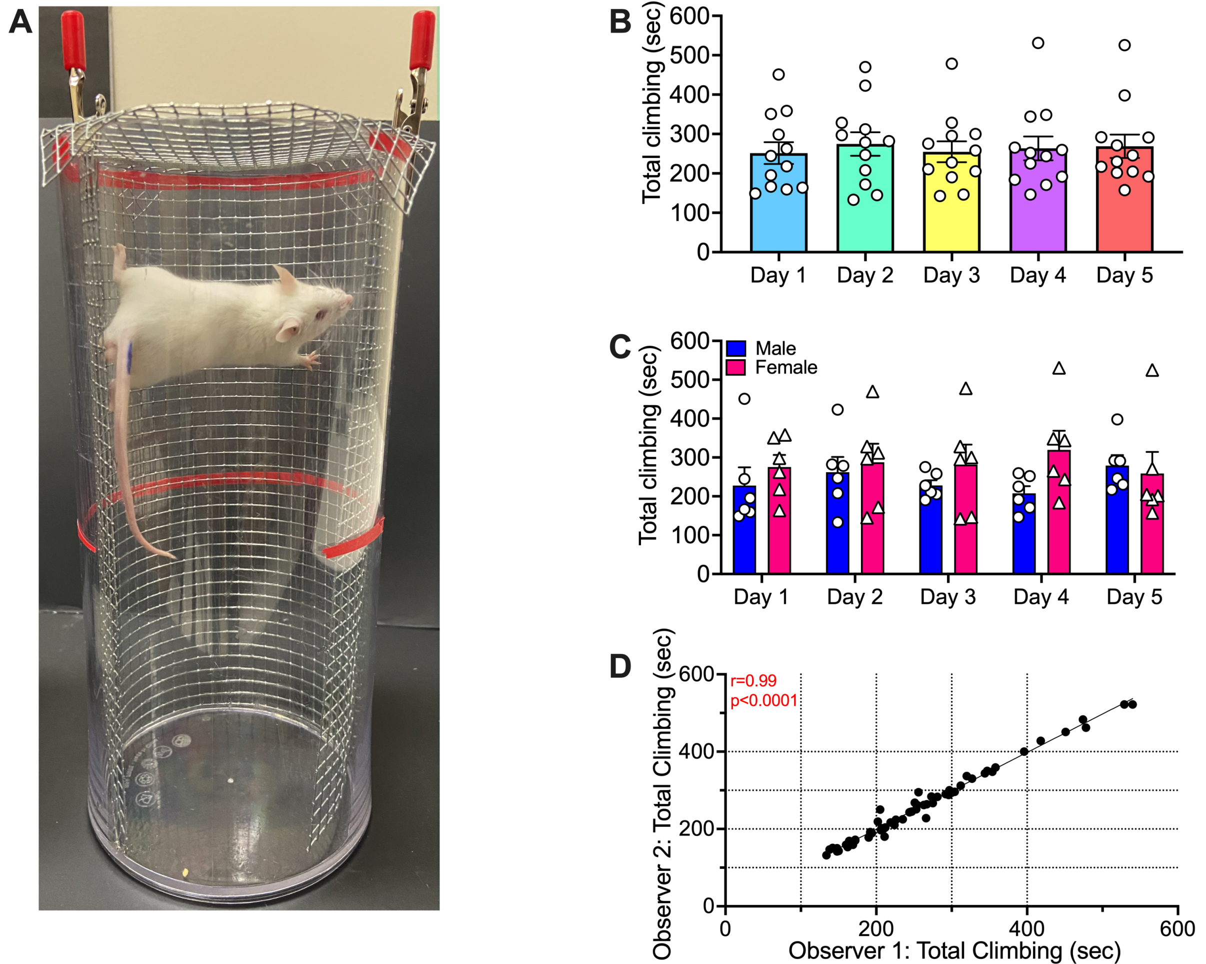


Figure 4.1: Climbing by mice during repeated testing. (A) The climbing apparatus. (B) Abscissa: Test Day. Ordinate: Time climbing in sec. Each bar shows mean ± SEM from 12 mice (6 male, 6 female), and points show data for individual mice. (C) Same data as in Panel B segregated by sex. (D) Inter-rater reliability of climbing times assigned by two different observers for all mice across all test days.

Table 4.1: Two-way ANOVA results with power analysis for Figure 4.1.

Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Day Main Effect	0.021	F (2.33, 23.30) = 0.22; p=0.8378	0.147	0.153	84
Sex Main Effect	0.124	F (1, 10) = 0.99; p=0.3423	0.375	0.651	17
Day x Sex Interaction	0.119	F (4, 40) = 1.36; p=0.2668	0.368	0.878	11

Subsequent studies were conducted in 16 different groups of mice. Each of these groups included one of three types of vehicle control: (1) IP water administered alone 10 min before the session (control for IP acid alone); (2) SC saline administered alone 20 min before the session (control for studies of drugs tested alone); or (3) both SC saline and IP water (control for drugs tested as pretreatments to IP acid). **Table 4.2** shows the mean±SEM climbing time for the vehicle control in each group, along with the number of mice in each group that failed to meet inclusion criteria during vehicle control testing. **Figure 4.2.A** compares climbing times across different vehicle controls. Climbing after SC saline alone was similar to climbing on Day 1 of the No Treatment group shown in Figure 1; however, IP water administered either alone or in conjunction with SC saline resulted in a significant decrease in climbing relative to SC saline alone [$F(2, 188) = 13.08$; $P<0.0001$]. Moreover, as shown in **Table 4.2**, only 3 mice climbed less than 60 sec after SC saline alone and thereby failed to meet inclusion criteria (3.4% of all mice tested), whereas 16 mice failed to meet inclusion criteria after IP water administered either alone or after SC saline (13% of all mice tested), and an additional 8 mice in these groups were excluded because IP 0.32% acid failed to produce a further decrease in climbing relative to the IP water control. To assess the stability of climbing within a vehicle control condition, **Figure 4.2.B** compares climbing in the seven different groups that received SC saline alone as their vehicle control, and there was no difference in climbing across these groups [$F(6, 77) = 0.9801$; $P=0.4446$]. Similarly, **Figure 4.2.C** compares climbing in the nine different groups that received IP water administered either alone or in conjunction with SC saline. Although there was a significant effect of group [$F(8, 98) = 2.176$; $P=0.0357$], post hoc analysis indicated that the only difference was between the

U69593+LA group and the FNT/NTX 10:1+LA group. Overall, then, baseline climbing was relatively stable between groups within a given vehicle-control condition, but relative to SC saline, IP water injections resulted in higher rates of exclusion due to low climbing times, reduced climbing time in mice that met inclusion criteria, and modest but significant variation between groups.

Figure 4.3 shows that IP acid produced a concentration-dependent depression of climbing that could be blocked by pretreatment with ketoprofen (a positive control analgesic) but not by U69593 (a negative control non-analgesic). Thus, **Figure 4.3.A** shows that IP acid produced a significant decrease in climbing at concentrations of 0.32 and 0.56% [$F(2.301, 25.31) = 12.50$; <0.0001], and the concentration of 0.32% was used for all subsequent studies. **Figure 4.3.B** shows that the nonsteroidal anti-inflammatory drug ketoprofen (10 mg/kg) administered alone had no effect on climbing, but it blocked IP acid-induced depression of climbing [$F(2.505, 27.55) = 15.38$; $P<0.0001$]. Conversely, the kappa opioid receptor agonist U69593 (0.1-1.0 mg/kg) produced a dose-dependent decrease in climbing when it was administered alone (**Figure 4.3.C**, [$F(2.853, 65.62) = 21.29$; $P<0.0001$]) and failed to block IP acid-induced depression of climbing (**Figure 4.3.D,** [$F(2.575, 28.33) = 1.130$; $P=0.3479$]). Statistical analysis of all **Figure 4.3** experiments segregated by sex is shown in **Table 4.3.** There were no sex x dose interactions for any experiment; however, there was a main effect of sex in the U69593+LA group [$F(1, 10) = 12.90$; $P=0.0049$], with males climbing less than females.

Table 4.2: Summary of vehicle control data and one-way ANOVA results for each group tested in the present study.

Treatment	Vehicle Condition	Mean Climbing (sec ± SEM) after Vehicle	Exclusions: Vehicle Climbing <60 sec	Exclusions: IP Acid Depression of Climbing <20%	One-way ANOVA
No treatment	none	251.5 ± 27.75 (day 1)	-	-	F (2.77, 30.42) = 0.21; P=0.8748
Lactic acid (LA)	IP H2O	159.2 ± 28.58	1M	-	F (2.30, 25.31) = 1.50; <0.0001
Ketoprofen ± LA	SC Sal + IP H2O	160.9 ± 15.21	3F	1M 2F	F (2.51, 27.55) = 15.38; P<0.0001
U69593	SC Sal	346.1 ± 44.25	-	-	F (2.85, 65.62) = 21.29; P<0.0001
Fentanyl	SC Sal	246.9 ± 26.88	1F	-	F (2.63, 28.98) = 26.27; P<0.0001
Buprenorphine	SC Sal	256.6 ± 43.47	-	-	F (2.06, 22.70) = 10.55; P=0.0005
Naltrexone	SC Sal	278.3 ± 29.64	-	-	F (2.16, 23.76) = 0.60; P=0.5715
FNT/NTX 10:1	SC Sal	245.4 ± 37.85	-	-	F (2.17, 23.87) = 28.79; P<0.0001
FNT/NTX 3.2:1	SC Sal	270.9 ± 14.53	-	-	F (2.79, 30.74) = 19.95; P<0.0001
FNT/NTX 1:1	SC Sal	236.1 ± 53.01	1M 1F	-	F (2.59, 28.52) = 1.16; P=0.3388
U69593 + LA	SC Sal + IP H2O	257.0 ± 48.13	2M	1F	F (2.58, 28.33) = 1.13; P=0.3479
Fentanyl + LA	SC Sal + IP H2O	170.0 ± 19.99	1M	-	F (2.08, 22.83) = 0.91; P=0.4184
Buprenorphine + LA	SC Sal + IP H2O	166.8 ± 20.71	1F	-	F (1.08, 11.91) = 0.87; P=0.3781
Naltrexone + LA	SC Sal + IP H2O	230.0 ± 38.27	2M 1F	-	F (1.61, 17.76) = 1.11; P=0.3381
FNT/NTX 10:1 + LA	SC Sal + IP H2O	208.9 ± 34.93	1F	-	F (1.62, 16.17) = 1.19; P=0.3199
FNT/NTX 3.2:1 + LA	SC Sal + IP H2O	172.8 ± 40.94	1M 3F	1F	F (2.14, 23.59) = 0.70; P=0.5178
FNT/NTX 1:1 + LA	SC Sal + IP H2O	201.8 ± 23.78	-	1M 2F	F (1.96, 21.51) = 2.63; P=0.0963

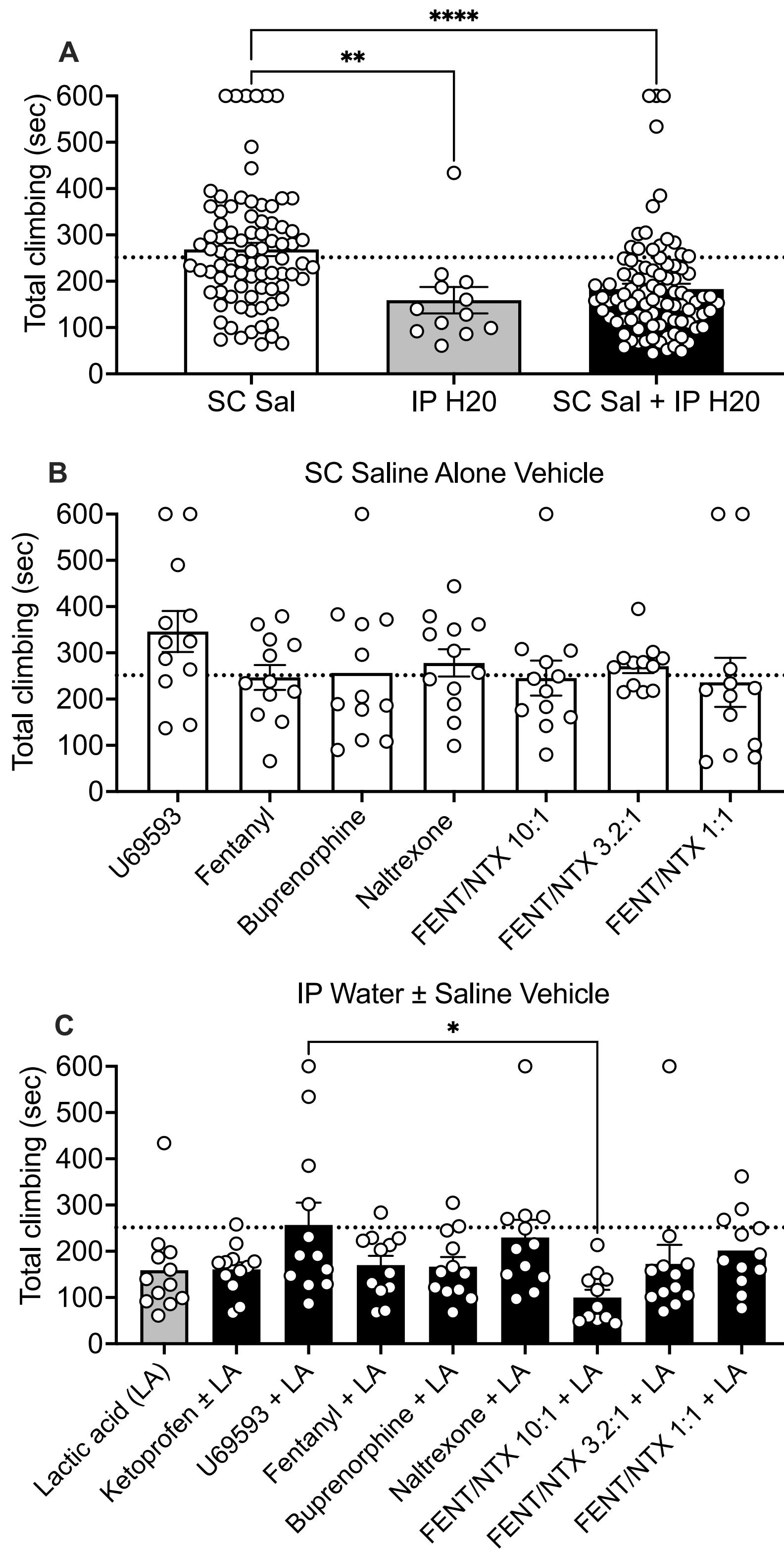


Figure 4.2: Effects of vehicle control conditions on climbing

Figure 4.2: Effects of vehicle control conditions on climbing [figure legend].

(A) Comparison of climbing times for the three different types of vehicle condition. Abscissa: Type of vehicle treatment: subcutaneous saline alone (SC Sal; N=84), intraperitoneal water alone (IP H20; N=12), and SC saline + IP water (N=95). (B) Comparison of climbing times for each group that received SC saline as the vehicle condition. Each group is identified by the drug or drug mixture tested in the group. (C) Comparison of climbing times for each group that received SC saline + IP water as the vehicle condition. Each group is identified by the drug or drug mixture tested as a pretreatment to IP lactic acid (LA) in the group. For all panels, the ordinate is total climbing time in sec, the dotted line shows the mean climbing time on Day 1 by the "No Treatment" group shown in Figure 1, bars show mean±SEM, and points show data for individual mice. Asterisks show a significant difference between groups as indicated by one-way ANOVA and Holm-Sidak post hoc test. *p<0.05, **p<0.01, ****p<0.0001.

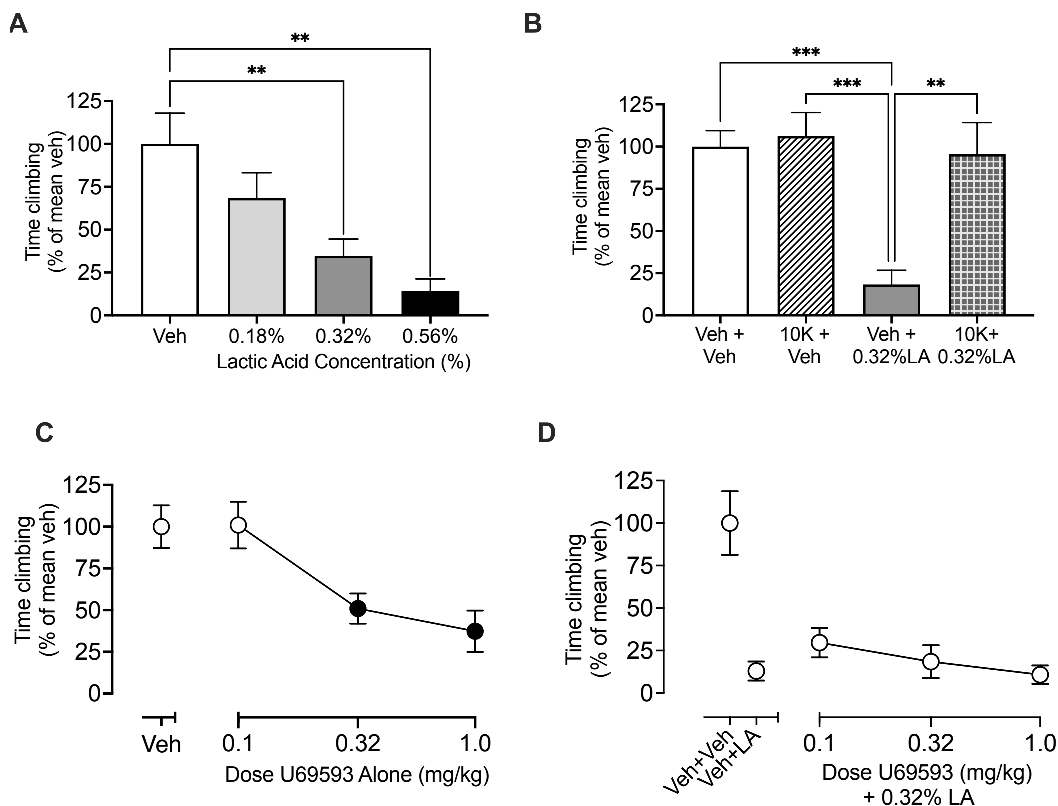


Figure 4.3: Effects of IP lactic acid, the positive control ketoprofen ± IP acid, and the negative control U69593 ± IP acid on climbing. (A) IP lactic acid concentration-effect curve. Abscissa: Concentration of lactic acid diluted in sterile water for IP injection. (B) Effects of ketoprofen ± IP acid. Abscissa: Treatment with SC Saline or 10 mg/kg ketoprofen ± IP water or 0.32% lactic acid. (C) Effects of the kappa opioid receptor agonist U69593 administered alone. Abscissa: U69593 dose in mg/kg. (D) Effects U69593 administered as a pretreatment to IP 0.32% lactic acid. Abscissa: Dose of the kappa opioid receptor agonist U69593 in mg/kg. For all panels, the ordinate is time climbing expressed as percentage of the mean climbing time after vehicle (Veh in A,C; Veh+Veh in B,D) in that group, and all bars and points show mean±SEM from 12 mice. Asterisks in panels A and B show a significant difference between groups. **$p<0.01$, ***$p<0.001$. Filled points in panel C indicate a significant difference from “Veh”, $p<0.05$. U69593 effects in Panel D were compared to Veh+LA by one-way ANOVA (Veh+Veh data not included in analysis), and results were not significant.

Table 4.3: Two-way ANOVA results with power analysis for Figure 4.3.

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Lactic acid (LA)	Dose Main Effect	0.578	F (2.38, 23.75) = 13.69; P<0.0001	1.170	1	10
	Sex Main Effect	0.103	F (1, 10) = 1.04; P=0.3315	0.339	0.563	20
	Dose x Sex Interaction	0.170	F (3, 30) = 2.04; P=0.1288	0.452	0.950	12
Ketoprofen	Dose Main Effect	0.021	F (1, 10) = 0.22; P=0.6522	0.147	0.152	96
	Sex Main Effect	0.289	F (1, 10) = 2.10; P=0.1779	0.639	0.977	8
	Dose x Sex Interaction	0.101	F (1, 10) = 1.12; P=0.3148	0.335	0.553	21
Ketoprofen + LA	Dose Main Effect	0.635	F (1, 10) = 17.43; P=0.0019	1.320	1	5
	Sex Main Effect	0.042	F (1, 10) = 0.27; P=0.6127	0.209	0.258	49
	Dose x Sex Interaction	0.062	F (1, 10) = 0.66; P=0.4360	0.257	0.363	33
U69593	Dose Main Effect	0.558	F (2.03, 20.27) = 12.63; P=0.0003	1.124	1	4
	Sex Main Effect	0.022	F (1, 10) = 0.35; P=0.5686	0.150	0.156	92
	Dose x Sex Interaction	0.049	F (3, 30) = 0.52; P=0.6725	0.228	0.390	29
U69593 + LA	Dose Main Effect	0.099	F (2.45, 24.45) = 1.09; P=0.3615	0.331	0.652	17
	Sex Main Effect	0.120	F (1, 10) = 12.90; P=0.0049	0.370	0.637	17
	Dose x Sex Interaction	0.060	F (3, 30) = 0.6342; P=0.5988	0.251	0.469	24

Figure 4.4 shows that single-molecule opioids (fentanyl, buprenorphine, naltrexone) and a graded series of fixed-proportion fentanyl/naltrexone mixtures (10:1, 3.2:1, 1:1 FENT/NTX) produced a dose- and efficacy-dependent decrease in climbing when they were administered alone but were ineffective to block IP acid-induced depression of climbing. Thus, **Figure 4.4.A** shows dose-dependent decreases in climbing by fentanyl [F(2.634, 28.98) = 26.27; P<0.0001] and buprenorphine [F(2.063, 22.70) = 10.55; P=0.0005] but not by naltrexone [F(2.160, 23.76) = 0.5958; P=0.5715], and **Figure 4.4.B** shows that IP acid-induced depression of climbing was not alleviated by fentanyl [F(2.075, 22.83) = 0.9139; P=0.4184], buprenorphine [F(1.083, 11.91) = 0.8709; P=0.3781], or naltrexone [F (1.614, 17.76) = 1.113; P=0.3381]. Similarly, **Figure 4.4.C** shows dose-dependent decreases in climbing with fentanyl/naltrexone mixtures of 10:1 FENT/NTX [F(2.170, 23.87) = 28.79; P<0.0001] and 3.2:1 FENT/NTX [F (2.794, 30.74) = 19.95; P<0.0001] but not by 1:1 FENT/NTX [F (2.592, 28.52) = 1.157; P=0.3388], and **Figure 4.4.D** shows that IP acid-induced depression of climbing was not significantly alleviated by the 10:1 mixture [F(1.617, 16.17) = 1.188; P=0.3199], 3.2:1 mixture [F (2.144, 23.59) = 0.6966; P=0.5178], or 1:1 mixture [F (1.956, 21.51) = 2.627; P=0.0963]. The 1:1 FENT/NTX mixture produced relatively high climbing times in some mice at the 0.032 mg/kg fentanyl/0.032 mg/kg naltrexone dose suggestive of an antinociceptive effect, but this effect did not meet the criterion for significance. Statistical analysis of all Figure 4 experiments segregated by sex is shown in **Table 4.4**. For most groups, there was not significant main effect of sex or sex x dose interaction. However, there was a main effect of sex effect in both the buprenorphine-alone group [F (1, 10) = 7.33; p=0.0220], and naltrexone+IP acid group [F (1, 10) = 6.15; P=0.0325], with males

climbing less than females in both groups. In addition, there was a significant sex x dose interaction in the buprenorphine-alone group [F (4, 40) = 3.74; p=0.0112], but post hoc testing did not indicate a significant effect of sex at any dose.

Figure 4.5 shows analysis of fentanyl/naltrexone-mixture data to indicate that MOR agonist-induced disruption of climbing has a very low efficacy requirement (i.e. climbing is highly sensitive to disruption by MOR agonists administered alone). **Figure 4.5.A** shows the linear regression of FENT/NTX mixture data used to determine an EP50 value as a measure of opioid efficacy to decrease climbing. **Figure 4.5.B** shows that the EP50 value for decreases in climbing is lower than EP50 values for a range of other previously published in vivo and in vitro effects produced by fentanyl/naltrexone mixtures.

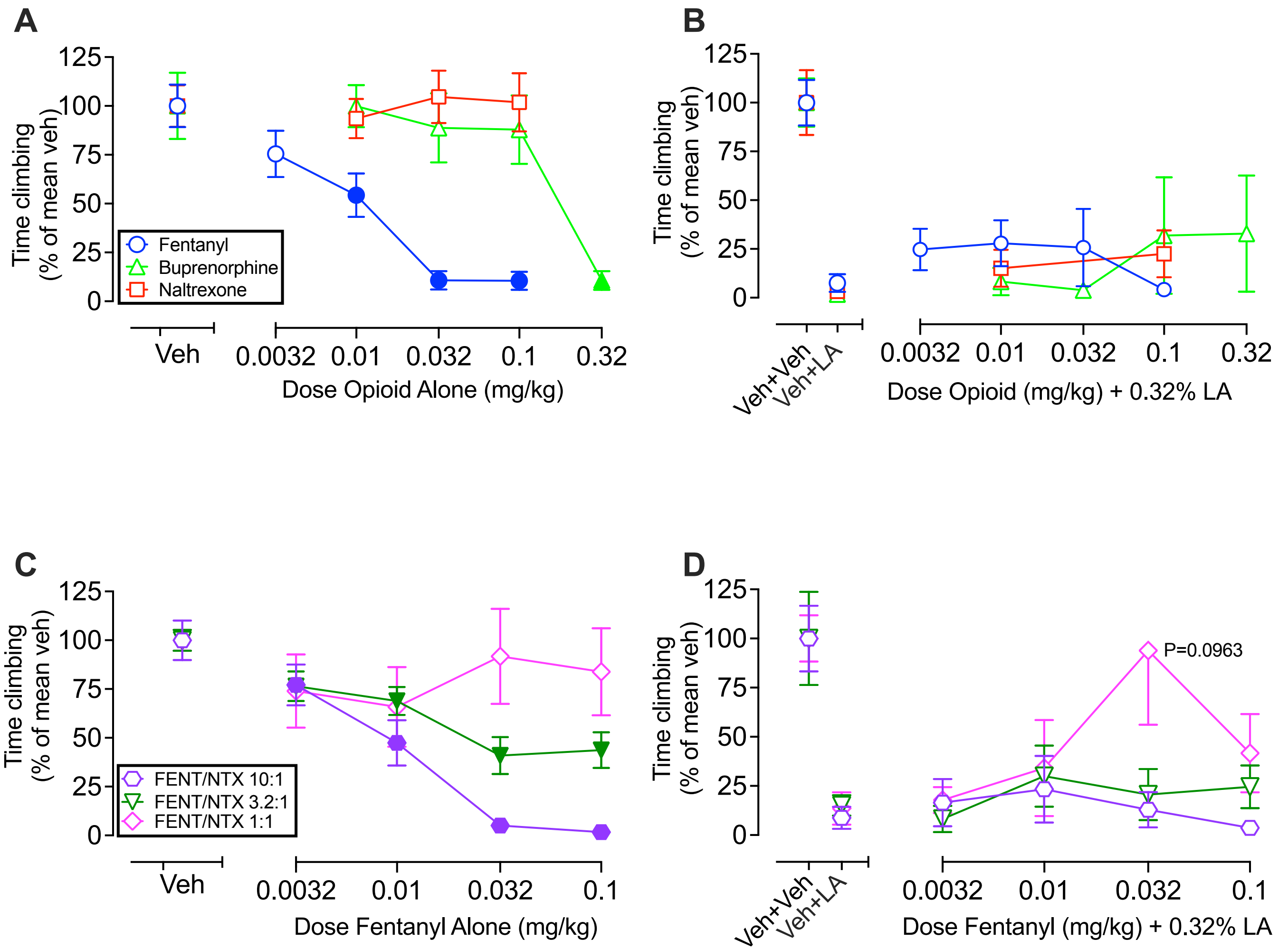


Figure 4.4: Effects of single-molecule opioids ± IP acid, and fentanyl/naltrexone (FENT/NTX) mixtures ± IP acid.

Figure 4.4: Effects of single-molecule opioids ± IP acid, and fentanyl/naltrexone (FENT/NTX) mixtures ± IP acid [figure legend].

(A) Effects of fentanyl, buprenorphine, and naltrexone administered alone. Abscissa: Dose of opioid alone in mg/kg. (B) Effects of fentanyl, buprenorphine, and naltrexone as pretreatments to IP 0.32% lactic acid. Abscissa: Dose of opioid in mg/kg. (C) Effects of 10:1, 3.2:1, and 1:1 FENT/NTX mixtures administered alone. Abscissa: Dose of fentanyl alone in mg/kg, with naltrexone dose varying according to the designated proportion. (D) Effects of 10:1, 3.2:1, and 1:1 FENT/NTX mixtures as pretreatments to IP 0.32% lactic acid. Abscissa: Dose of fentanyl in mg/kg, with naltrexone dose varying according to the designated proportion. For all panels, the ordinate is time climbing expressed as percentage of the mean climbing time after vehicle (Veh in A,C; Veh+Veh in B,D) in that group, and all bars and points show mean±SEM from 12 mice, except for FENT/NTX 10:1 + IP lactic acid (N=11). Filled points in panels A and C indicate a significant difference from "Veh", $p<0.05$. Drug effects in Panels B and D were compared to Veh+LA by one-way ANOVA (Veh+Veh data not included in analysis), and no drug effects were significant. Panel D shows that the one-way ANOVA for the 1:1 mixture approached the criterion for significance ($P=0.0963$), and a Dunnett's post hoc test indicated a P value of 0.1724 in comparing Veh+LA with the 0.032 mg/kg Fentanyl/0.032 mg/kg Naltrexone dose.

Table 4.4: Two-way ANOVA results with power analysis for Figure 4.4.

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Fentanyl	Dose Main Effect	0.722	F (2.68, 26.76) = 26.03; p<0.0001	1.613	1	3
	Sex Main Effect	0.174	F (1, 10) = 3.27; p=0.1006	0.459	0.818	12
	Dose x Sex Interaction	0.082	F (4, 40) = 0.90; p=0.4736	0.299	0.695	15
Buprenorphine	Dose Main Effect	0.568	F (2.50, 25.00) = 13.17; p<0.0001	1.148	1	3
	Sex Main Effect	0.351	F (1, 10) = 7.33; p=0.0220	0.736	0.995	7
	Dose x Sex Interaction	0.272	F (4, 40) = 3.74; p=0.0112	0.611	0.999	5
Naltrexone	Dose Main Effect	0.055	F (2.08, 20.76) = 0.58; P=0.5761	0.240	0.346	33
	Sex Main Effect	0.156	F (1, 10) = 0.39; p=0.5427	0.430	0.766	14
	Dose x Sex Interaction	0.063	F (3, 30) = 0.67; p=0.5789	0.258	0.490	23
FENT/NTX 10:1	Dose Main Effect	0.744	F (1.95, 19.53) = 29.07; P<0.0001	1.705	1	4
	Sex Main Effect	0.624	F (1, 10) = 1.91; P=0.1970	1.289	1	5
	Dose x Sex Interaction	0.099	F (4, 40) = 1.11; P=0.3661	0.333	0.796	13
FENT/NTX 3.2:1	Dose Main Effect	0.687	F (2.59, 25.85) = 21.97; p<0.0001	1.482	1	4
	Sex Main Effect	0.118	F (1, 10) = 0.75; p=0.4079	0.366	0.628	18
	Dose x Sex Interaction	0.174	F (4, 40) = 2.11; p=0.0971	0.460	0.980	8
FENT/NTX 1:1	Dose Main Effect	0.105	F (2.62, 26.18) = 1.17; P=0.3357	0.342	0.677	16
	Sex Main Effect	0.0002	F (1, 10) = 0.0007; p=0.9783	0.015	0.051	>100
	Dose x Sex Interaction	0.102	F (4, 40) = 1.14; p=0.3536	0.337	0.807	13
Fentanyl + LA	Dose Main Effect	0.084	F (2.10, 20.95) = 0.91; P=0.4218	0.302	0.491	23

	Sex Main Effect	0.040	F (1, 10) = 2.04; p=0.1837	0.203	0.247	51
	Dose x Sex Interaction	0.088	F (4, 40) = 0.96; p=0.4399	0.310	0.728	15
Buprenorphine + LA	Dose Main Effect	0.080	F (1.08, 10.78) = 0.87; P=0.3812	0.294	0.321	38
	Sex Main Effect	0.073	F (1, 10) = 1.17; P=0.3045	0.281	0.421	28
	Dose x Sex Interaction	0.085	F (4, 40) = 0.93; P=0.4591	0.304	0.710	15
Naltrexone + LA	Dose Main Effect	0.105	F (1.37, 13.65) = 1.17; P=0.3186	0.342	0.546	21
	Sex Main Effect	0.154	F (1, 10) = 6.15; P=0.0325	0.426	0.758	14
	Dose x Sex Interaction	0.135	F (2, 20) = 1.56; P=0.2340	0.395	0.801	13
FENT/NTX 10:1 + LA	Dose Main Effect	0.129	F (1.52, 13.63) = 1.33; P=0.2877	0.384	0.554	19
	Sex Main Effect	0.197	F (1, 9) = 1.16; P=0.3100	0.496	0.832	11
	Dose x Sex Interaction	0.102	F (4, 36) = 1.03; P=0.4065	0.338	0.763	13
FENT/NTX 3.2:1 + LA	Dose Main Effect	0.063	F (2.01, 20.08) = 0.672; P=0.5226	0.259	0.367	31
	Sex Main Effect	0.005	F (1, 10) = 0.109; P=0.7486	0.074	0.075	>100
	Dose x Sex Interaction	0.057	F (4, 40) = 0.604; P=0.6618	0.246	0.502	22
FENT/NTX 1:1 + LA	Dose Main Effect	0.208	F (2.02, 20.19) = 2.63; P=0.0962	0.513	0.921	10
	Sex Main Effect	0.045	F (1, 10) = 0.752; P=0.4062	0.216	0.274	45
	Dose x Sex Interaction	0.092	F (4, 40) = 1.01; P=0.4131	0.318	0.753	14

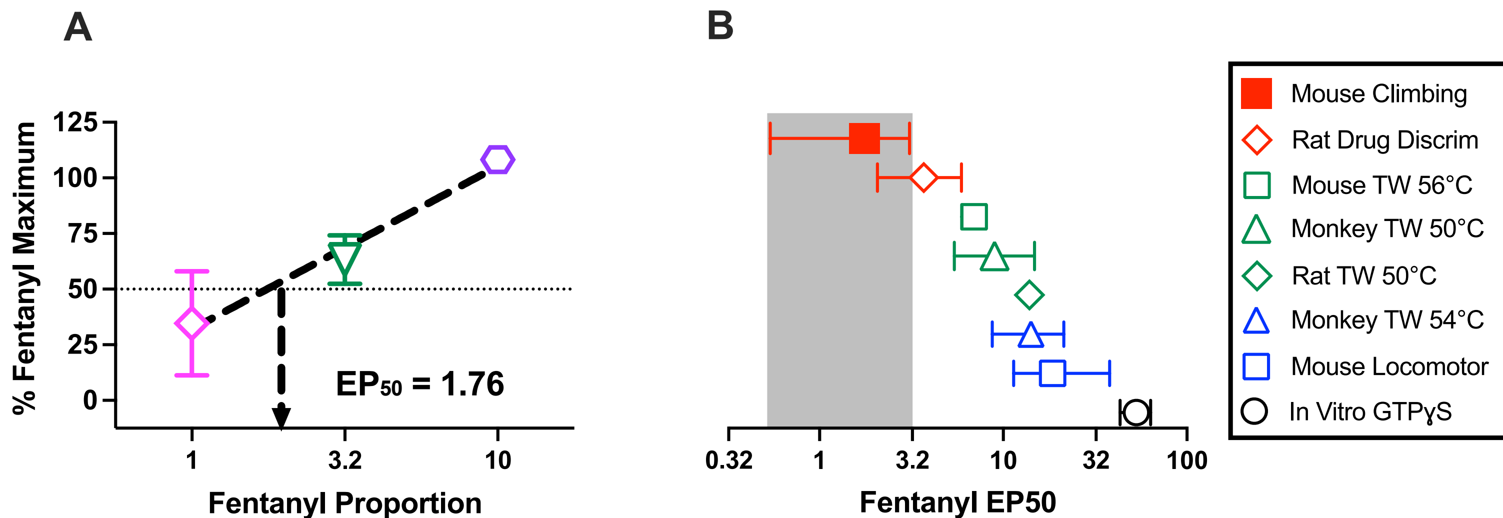


Figure 4.5: Efficacy requirement for Fentanyl/Naltrexone mixtures to decrease climbing. (A) Determination of EP50 value as a measure of opioid efficacy to decrease climbing. Abscissa: Proportion of fentanyl in the mixture. Ordinate: Maximum effect of each mixture in Figure 4C expressed as a percentage of the maximum effect of fentanyl alone in Figure 4A. The efficacy requirement of the mixtures to decrease climbing can be determined by linear regression and expressed as the EP50 value, defined as the proportion of fentanyl sufficient to produce a maximum effect equal to 50% of the fentanyl-alone maximum. Points show mean±SEM of N=11-12 mice. (B) Comparison of EP50 values across multiple endpoints determined either in vivo (in the designated species) or in vitro (in cultured cells expressing the mouse mu opioid receptor) as reported in previous publications. Error bars show 95% confidence limits. Drug Discrim=drug discrimination; TW X°C=warm-water tail-withdrawal assay of thermal antinociception with a water temperature of X°C; GTPγS=assay of agonist-stimulated GTPγS binding.

4.4. Summary

This study developed a novel assay of climbing behavior in mice and evaluated the utility of climbing as a behavioral endpoint for preclinical research on drug effects in the absence or presence of acute pain. There were three main findings. First, under baseline conditions, mice engaged in high levels of climbing that were relatively stable both across repeated testing within a group of mice and between different groups of mice. Second, climbing was depressed by IP injection of dilute acid as a visceral noxious stimulus, and this IP acid-induced depression of climbing could be blocked by the NSAID analgesic positive control ketoprofen but not by the KOR agonist negative control U69593. These findings suggest that climbing may be specifically useful as one endpoint for studies to examine effectiveness of candidate analgesics to alleviate pain-related behavioral depression. Lastly, climbing was dose-dependently reduced by MOR agonists, and analysis of results with fentanyl/naltrexone mixtures indicated that climbing in mice is more sensitive than many other behavioral endpoints to disruption by MOR agonists. These findings suggest that climbing may be especially useful for sensitive detection of undesirable motor effects of MOR agonists; however, this high sensitivity to direct effects of MOR agonists also appeared to prevent expression of an analgesic effect. Thus, climbing as assessed here illustrates the limits of MOR agonist effectiveness to restore pain-depressed behavior, and this procedure may not be useful to evaluate novel MOR agonists as candidate analgesics.

Chapter Five

Efficacy as a Determinant of Mu Opioid Receptor (MOR) Analgesic Effects in a Novel Assay of Pain-Depressed Behavior in Mice. II. Effects of Novel Low-Efficacy MOR Agonists

5.1. Introduction

A main goal of the work presented in this dissertation was to use an assay of pain-depressed behavior to study the effects of low-efficacy opioids as a potential class of novel analgesics. **Chapter 4** discussed the validation process of one novel behavior, the mouse climbing assay. The results from that chapter indicated that climbing is a very sensitive assay to opioid motor disruption effects, which prevented alleviation of the pain-depressed behavior. Thus, two goals moving forward were (1) to establish and validate a different assay of pain-depressed behavior that was less sensitive to opioid disruption, and (2) to study the effects of the three strategies described previously to manipulate and study MOR efficacy. Strategy one involved studying the effects of clinically available single-molecule opioids (**Chapters 2** and **4**), strategy two involved studying the effects of fentanyl/naltrexone mixtures (**Chapters 2** and **4),** and the final strategy involved studying the effects of novel single-molecule opioids (**Chapter 3**).

To address the first of these goals, we developed a novel assay, which we call the "locomotor + barrier" procedure, that combines evaluation of horizontal and vertical activity in mice. As described in more detail in below in Methods, the procedure uses a behavioral chamber with two compartments separated by a doorway that is obstructed by a wire-mesh barrier. From this assay, two behavioral endpoints are measured: (1) "Crosses" defined as the number of crosses between the compartments, which requires

mice to rear and surmount the vertical barrier in the doorway, and (2) “Movement” defined as the total number of beam breaks in each individual compartment, which requires only horizontal locomotor activity. Previous work from our lab (unpublished) has worked on validating this assay and studying the effects of 2 out of 3 of the strategies to manipulate MOR efficacy.

This chapter will focus on the effects of the novel single-molecule opioids as described previously in **Chapter 3** (Chambers et al., 2022; Gutman et al., 2020) (Lutz et al., 2023; *In Press*, Tom Prinsenzano; personal communication), in the novel locomotor + barrier assay and not the initial validation stages. However, a brief discussion on the validation stage will be described below to provide the rationale for the methods used in this chapter. The initial validation process involved manipulating (1) the height of the wire-mesh barrier in the doorway (0-1.5 inches; 0-3.81 cm) that is placed between both locomotor compartments and (2) manipulating the concentration (0-1.0%) and pretreatment time (5-160 min) of IP lactic acid administered as a pain stimulus. Based on results from this study, the 1.0-inch (2.54 cm) barrier height and 0.56% of IP lactic acid was used in subsequent studies. The next step focused on evaluating the effects of (1) clinically available single-molecule opioids that varied in MOR efficacy, (2) a series of fentanyl/naltrexone mixtures, and (3) additional positive and negative controls on behavior alone and alleviation of pain-depressed behavior. Results indicated that relatively high MOR efficacy was required to reduce behavior in this procedure, providing evidence that the behavioral endpoints in this procedure were less sensitive than climbing to disruption by MOR agonists. Additionally, the opioids produced an efficacy-dependent effect to alleviate pain-depressed behavior, and importantly, low- to intermediate-efficacy

MOR agonists or mixtures that did not decrease behavior when administered alone were effective to alleviate IP acid-induced behavioral depression. Thus, these results supported the potential of low-efficacy MOR agonists to produce significant antinociceptive effects with fewer side effects than high-efficacy MOR agonists. The other positive control tested (the NSAID ketoprofen) was also effective; however, a series of negative controls (i.e. diazepam, U69593, psilocybin) were not effective in alleviating the pain-depressed behavior. These results provided the foundation and rationale for the studies conducted in this chapter, and we hypothesized that effectiveness of the novel opioids to alleviate the pain-depressed behavior would be dependent on the MOR efficacy of the opioid, with greater effects observed with intermediate- to low-efficacy opioids at doses that do not cause motor disruption on their own.

5.2. Methods.

5.2.1 Subjects

Male and female ICR mice (Envigo, Frederick, MD) were 6–8 weeks old upon arrival to the laboratory, where they were single-housed in cages with corncob bedding (Envigo), a “nestlet” composed of pressed cotton (Ancare, Bellmore, NY), a cardboard tube for enrichment, and ad libitum access to water and food (Teklad LM-485 Mouse/Rat Diet; Envigo). Cages were mounted in racks in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed during the light phase of the daily light/dark cycle beginning at least 1 week after arrival at the laboratory. Ethical animal-use protocols were approved by the Virginia

Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

5.2.2. Apparatus

Locomotor activity was assessed in plexiglass and metal test boxes housed in sound-attenuating chambers (Med Associates, St. Albans, VT) and located in a procedure room separate from the housing room. Each box had two adjacent compartments (16.8 × 12.7 cm^2 floor area × 12.7 cm high) separated by a central wall. One compartment had black walls with a bar floor, and the other compartment had white walls with a wire-mesh floor. Additionally, each compartment had a clear plexiglass lid fitted with a house light that illuminated during experimental sessions as well as six photobeams arranged at 3-cm intervals across the long wall and 1 cm above the floor and monitored by a microprocessor operating Med Associates software. The wall separating the two compartments contained a central door (5 cm wide x 6 cm high), and the lower 1-inch (2.54 cm) of the door was obstructed by a wire-mesh barrier that had to be surmounted for mice to cross back and forth between the two compartments.

5.2.3. Experimental Procedure

This study was conducted by two different investigators (one male and one female), each of whom tested different treatments. As an initial test of inter-investigator reliability, both investigators tested different concentrations of lactic acid (0.18-0.56%) to determine if there was an effect of investigator on expression of IP acid-induced behavioral depression. Because no investigator effect was determined, the IP acid concentration for all following experiments was 0.56% administered 5-min before 15-min

experimental sessions with a barrier height of 1 inch (2.54 cm). Each test drug was then evaluated under two conditions to assess drug effects in the absence and presence of IP acid. First, vehicle and a range of drug doses was tested alone, with vehicle or drug being administered SC 30 min before the 15-min test session. Second, vehicle and a range of drug doses was tested as a pretreatment to IP acid. For these experiments, the test drug or its vehicle was administered SC 30 min before the 15-min session, and 0.56% lactic acid was administered IP 5 min before the session. The drugs and dose ranges were as follows: buprenorphine (0.01-0.32 mg/kg), DC-01-128.1 (0.1-3.2 mg/kg), DC-001-76.2 (0.1-3.2 mg/kg), EWB-3-14 (0.1-3.2 mg/kg), JL-02-0039 (0.32-10 mg/kg), DC-001-76.1 (0.32-10 mg/kg), EG-1-203 (1.0-32 mg/kg), EG-1-230 (1.0-32 mg/kg). The novel opioids were all MOR selective and varied in their efficacy to inhibit adenylate cyclase as discussed in **Chapter 3** (Chambers et al., 2022; Gutman et al., 2020) (Lutz et al., 2023; *In Press*, Tom Prinsenzano; personal communication). As a reminder of their relative efficacies, **Table 5.1** again shows the drugs and their E_{max} values from the in vitro assay of adenylate cyclase inhibition. For all experiments throughout the study, each treatment was tested in a group of 12 mice (6 female and 6 male), and each mouse was tested only once. Thus, for example, each dose of a given test drug was examined in a different group of 12 mice, and multiple groups of mice were used to determine effects of the multiple doses contributing to each dose-effect curve. The only exception was the DC-001-76.2 + IP acid group. One mouse assigned to this group died before experiments began, so this group contained only 11 mice (6 male, 5 female). In general, cohorts of mice were received from the vendor each week, acclimated to the housing facility for the remainder of that week, randomly assigned to the treatment conditions for testing during the

following week, and euthanized at the end of the test week. The experimenters were not blind to treatment conditions because data collection was automated by computer software, and all data from all mice were submitted to analysis as described below. No data were excluded.

Table 5.1: Emax values of In Vitro cAMP inhibition

Drug name	%Emax	Reference
	MOR Emax (In Vitro cAMP)	
Morphine	102.1	Chambers et al 2022
DC-01-128.1	101	Chambers et al 2022
Buprenorphine	100.3	Tom Prinsenzano (Personal communication)
DC-01-0076.2	94.7	Chambers et al 2022
EWB-3-14	91	Tom Prinsenzano (Personal communication)
JL-02-0039	85.0	Lutz et al 2023; *In Press*
DC-01-0076.1	67.3	Chambers et al 2022
EG-1-203	63.3	Gutman et al 2020
EG-1-230	33.5	Gutman et al 2020
Naltrexone	29.6	Chambers et al 2022

5.2.4. Data and Statistical Analysis

Data analysis for each session in each mouse focused on two dependent measures of activity in the 2-compartment locomotor apparatus: (1) “Crosses” defined as the number of crosses between the compartments, which required mice to rear and surmount the vertical barrier in the doorway, and (2) “Movement” defined as the total number of beam breaks in each individual compartment, which required only horizontal locomotor activity. Results were averaged across mice within a given treatment and submitted to analysis that proceeded in three steps as we have described previously for preclinical studies that include both sexes but are not intended to examine sex as the primary variable of interest (Diester et al., 2019; Santos et al., 2022). First, data for a given manipulation were pooled across sexes and analyzed by one-way ANOVA. A significant ANOVA was followed by Dunnett’s post hoc test to compare test treatments with vehicle treatment. Second, data were segregated by sex and analyzed by two-way ANOVA, with sex as one of the variables. A significant main effect of sex or sex x treatment interaction was followed by a Holm-Sidak post hoc test. Lastly, two-way ANOVA results were submitted to post hoc power analyses to calculate the Cohen’s f effect size, achieved power (1 - β), and the total number of animals predicted as necessary to achieve power ≥ 0.8. This post hoc power analysis was included to provide guidance for future studies that might investigate sex as a primary variable of interest. Prism 9.0 (GraphPad) was used for all ANOVAs, and the criterion for significance was $p<0.05$. G*power (Faul et al., 2007) was used for all post hoc power analyses.

5.2.5. Drugs

(±) Buprenorphine HCl was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). DC-01-128.1, DC-01-0076.2, EWB-3-14, JL-02-0039, DC-01-0076.1, EG-1-203, EG-1-230 HBr were provided by Dr. Kenner Rice and his colleagues in the Drug Design and Synthesis Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism (Bethesda, MD). Buprenorphine was dissolved in sterile saline and all other compounds were dissolved in a vehicle consisting of 5% ethanol, 5% emulphor, and 90% saline. Doses were calculated using the salt or free-base form of each drug described above and were administered subcutaneously (SC) in a volume of 10 ml/kg. Lactic acid (Sigma Aldrich) was diluted in sterile water and administered IP. All solutions were administered in a volume of 10 ml/kg.

5.3. Results

Figure 5.1. A-B shows the effects of IP acid tested by two different investigators on crosses and movement. Data were segregated by investigator and analyzed by two-way ANOVA **(Table 5.2).** This analysis indicated that, for crosses, there was a main effect of acid concentration, but no main effect of investigator, and no investigator x concentration interaction (**Figure 5.1.A**). Similarly, for movement, there was a main effect of acid concentration, but no main effect of investigator, and no investigator x dose interaction (**Figure 5.1.B**).

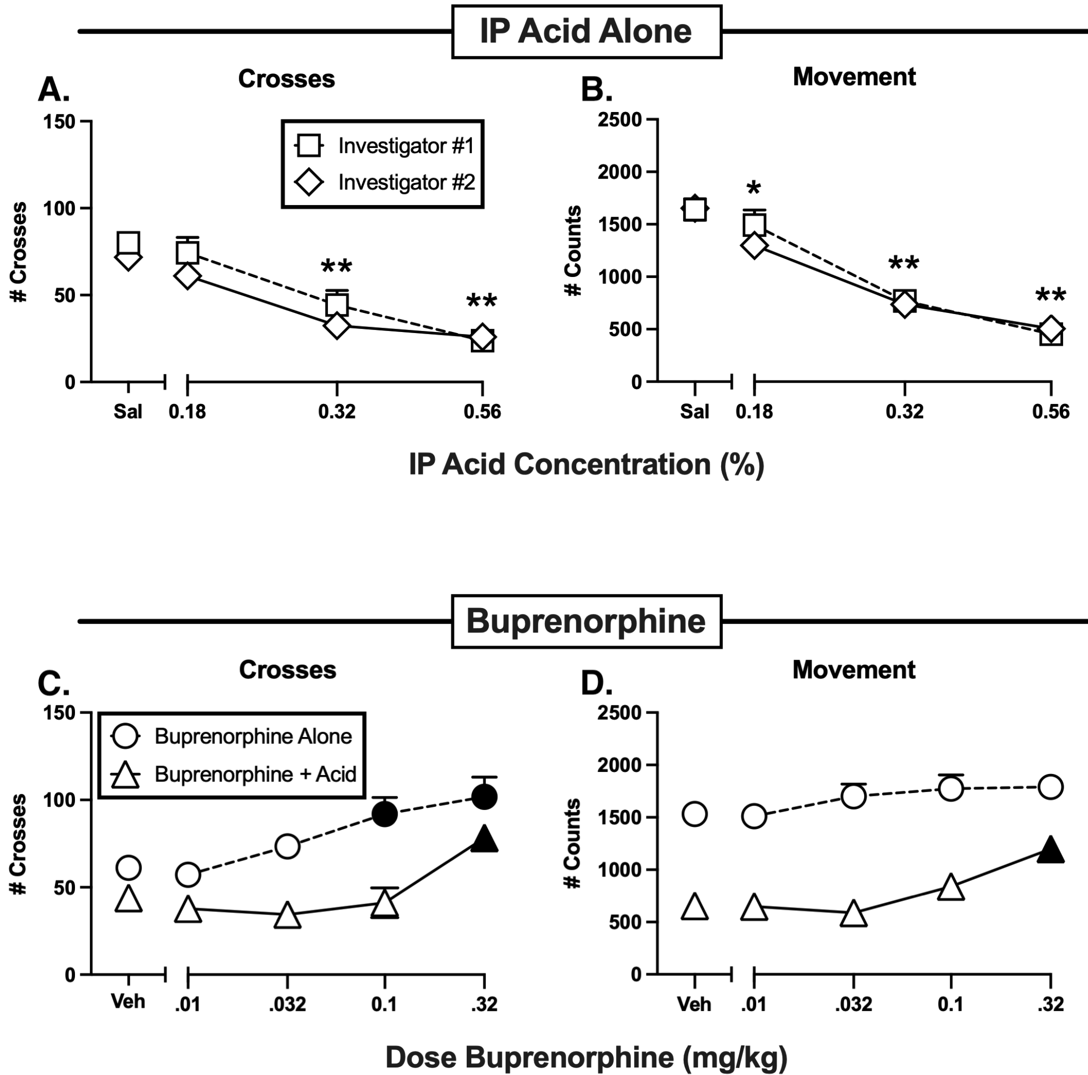


Figure 5.1: Shows the effects of IP lactic acid and buprenorphine on crosses and movement. Effects of IP acid on crosses (A) and movement (B). Abscissa: Concentration of lactic acid diluted in sterile water for IP injection. Buprenorphine effects alone and in the presence of 0.56% IP lactic acid in (C) crosses and (D) movement. Abscissa: Dose of buprenorphine in mg/kg. For panels A and C, the ordinate is number of crosses and for panels B and D the ordinate is number of counts.

Table 5.2: Two-way ANOVA results for Figure 5.1. A-B.

Treatment	Dependent measure	F statistic, p value
Crosses Lactic acid	Dose Main Effect	F (3, 88) = 31.17; P<0.0001
	Investigator Main Effect	F (1, 88) = 3.280; P=0.0735
	Dose x Investigator Interaction	F (3, 88) = 0.6481; P=0.5862
Movement Lactic acid	Dose Main Effect	F (3, 88) = 63.27; P<0.0001
	Investigator Main Effect	F (1, 88) = 0.3401; P=0.5612
	Dose x Investigator Interaction	F (3, 88) = 0.6258; P=0.6002

Once IP acid-induced depression of crosses and movement had been confirmed for both investigators, we next evaluated the effects of eight opioids administered alone and as a pretreatment to 0.56% IP acid. Buprenorphine was tested first as a clinically available intermediate-efficacy MOR agonist for comparison to effects of the seven novel opioids. **Figure 5.1.C**, **5.1.D** – **Figure 5.4** shows the effects on crosses and movement of buprenorphine and the novel single-molecule opioids when tested alone and the in presence of 0.56% IP acid. Each panel shows the effects of one drug administered alone (circles) and as a pretreatment to IP acid (triangles) on either crosses (left panels) or movement (right panels). **Table 5.3** shows one-way ANOVA results for data pooled across males and females. **Figure 5.1. C - D** and **Table 5.3** show that, when tested alone, buprenorphine significantly increased the number of crosses but did not significantly affect movement. When administered as a pretreatment to IP acid, buprenorphine at a dose of 0.32 mg/kg significantly alleviated the IP acid-induced depression of both crosses and movement.

Figure 5.2 and **Table 5.3** show the effects of three higher efficacy novel opioids. **Figure 5.2 A – B** show that, when tested alone, DC-1-128.1 significantly decreased the number of crosses and movement. When administered as a pretreatment to IP acid, DC-01-128.1 did not significantly alleviate the IP acid-induced depression of crosses, but significantly alleviated the IP acid-induced depression of movement at doses of 0.32 and 0.56 mg/kg. **Figure 5.2 C - D** show that, when tested alone, DC-01-0076.2 significantly increased the number of crosses but had no effect on movement. When administered as a pretreatment to IP acid, DC-01-0076.2 significantly alleviated the IP acid-induced depression of crosses at a dose of 1.0 mg/kg, and movement at doses of 1.0 and 3.2

mg/kg. **Figure 5.2 E - F** show that, when tested alone, EWB-3-14 significantly increased the number of crosses and movement. When administered as a pretreatment to IP acid, EWB-3-14 significantly alleviated the IP acid-induced depression of crosses at doses of 1.0 and 3.2 mg/kg, and movement at doses of 0.32, 1.0, and 3.2 mg/kg.

Figures 5.3 and **5.4** and **Table 5.3** show the effects of the four lower efficacy novel opioids. **Figure 5.3. A – B** show that, when tested alone, JL-02-0039 significantly increased the number of crosses but had no effect on movement. When administered as a pretreatment to IP acid, JL-02-0039 significantly alleviated the IP acid-induced depression of crosses at a dose of 5.6 mg/kg, and movement at doses of 3.2, 5.6, and 10 mg/kg. **Figure 5.3. C – D** show that, when tested alone, DC-01-0076.1 did not have an effect on crosses or movement. When administered as a pretreatment to IP acid, DC-01-0076.1 significantly alleviated the IP acid-induced depression of crosses and movement at doses of 3.2, and 10 mg/kg. **Figure 5.3. E – F** show that, when tested alone, EG-1-203 did not have an effect on crosses or movement. When administered as a pretreatment to IP acid, EG-1-203 did not significantly alleviate the IP acid-induced depression of crosses and movement at any of the doses tested. **Figure 5.4. A – B** show that, when tested alone, EG-1-230 did not have an effect on crosses or movement. When administered as a pretreatment to IP acid, EG-1-230 did not significantly alleviate the IP acid-induced depression of crosses and movement at any of the doses tested.

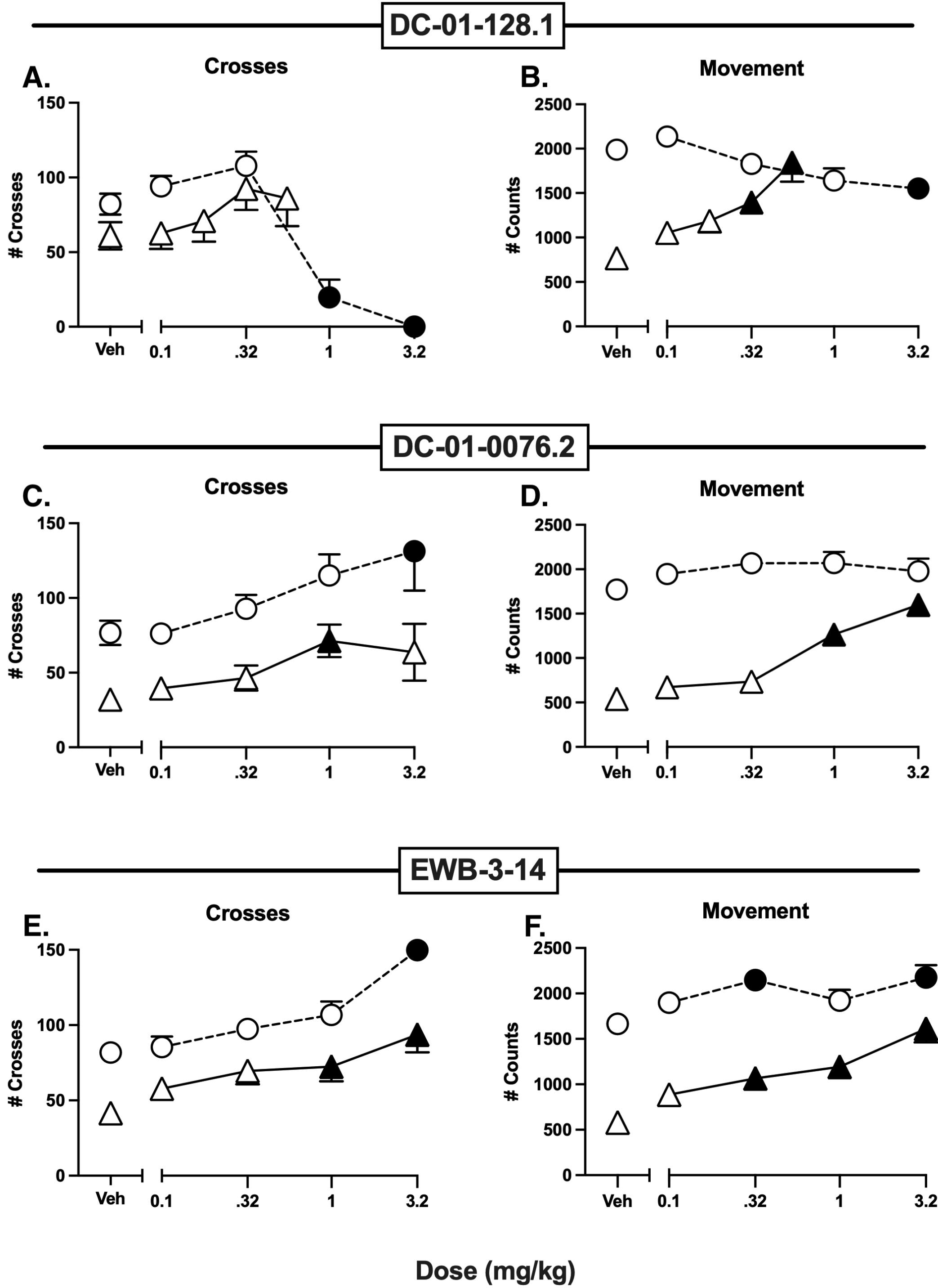
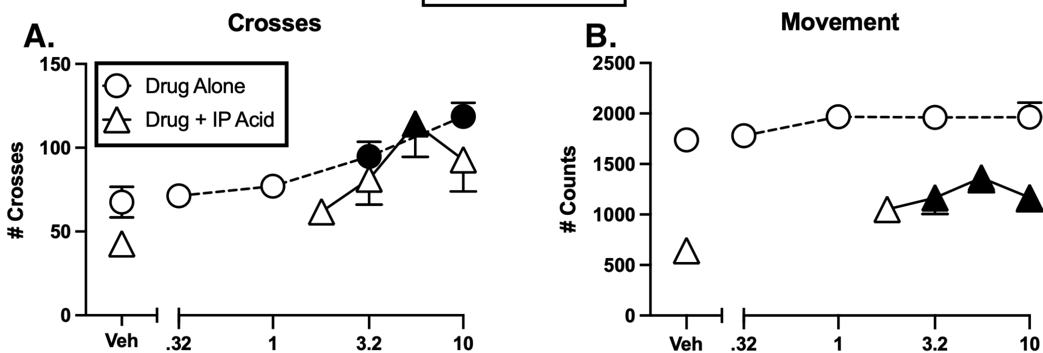
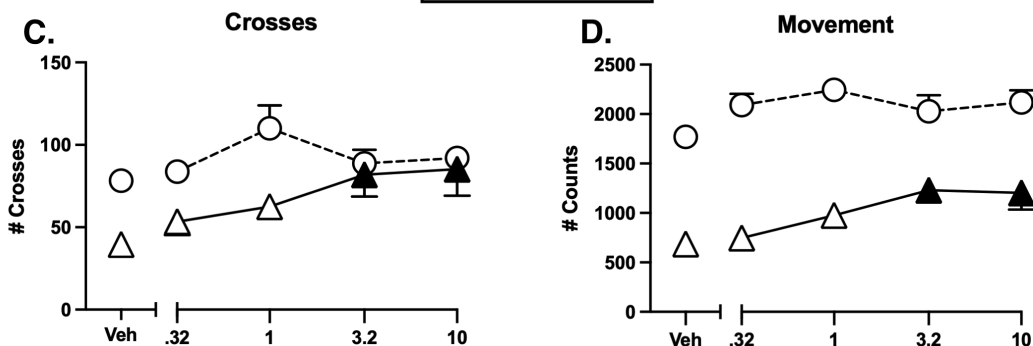


Figure 5.2. A - F Shows the effects of high-efficacy opioids.

Figure 5.2. A - F Shows the effects of high-efficacy opioids [figure legend]. DC-01-128.1 effects alone and in the presence of 0.56% IP lactic acid in (A) crosses and (B) movement. DC-01-0076.2 effects alone and in the presence of 0.56% IP lactic acid in (C) crosses and (D) movement. EWB-3-14 effects alone and in the presence of 0.56% IP lactic acid in (E) crosses and (F) movement. Abscissa: Dose of opioid in mg/kg. For panels A, C, and E the ordinate is number of crosses and for panels B, D, and F the ordinate is number of counts.





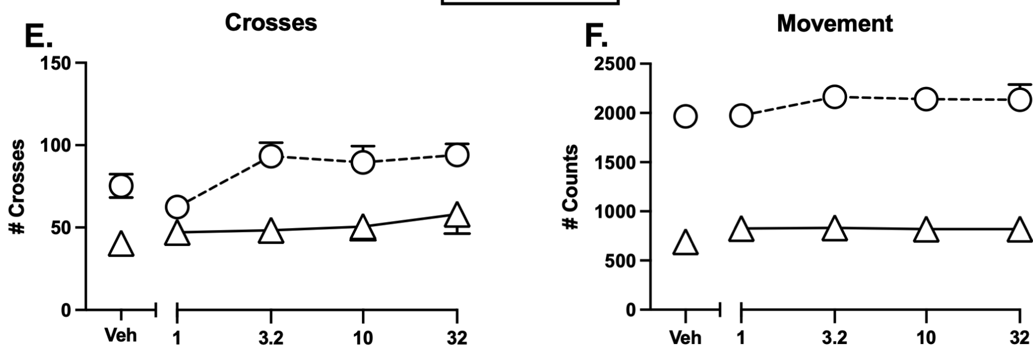


Figure 5.3. A - F Shows the effects of intermediate-efficacy opioids.

Figure 5.3. A - F Shows the effects of intermediate-efficacy opioids [figure legend]. JL-02-0039 effects alone and in the presence of 0.56% IP lactic acid in (A) crosses and (B) movement. DC-01-0076.1 effects alone and in the presence of 0.56% IP lactic acid in (C) crosses and (D) movement. EG-1-203 effects alone and in the presence of 0.56% IP lactic acid in (E) crosses and (F) movement. Abscissa: Dose of opioid in mg/kg. For panels A, C, and E the ordinate is number of crosses and for panels B, D, and F the ordinate is number of counts.

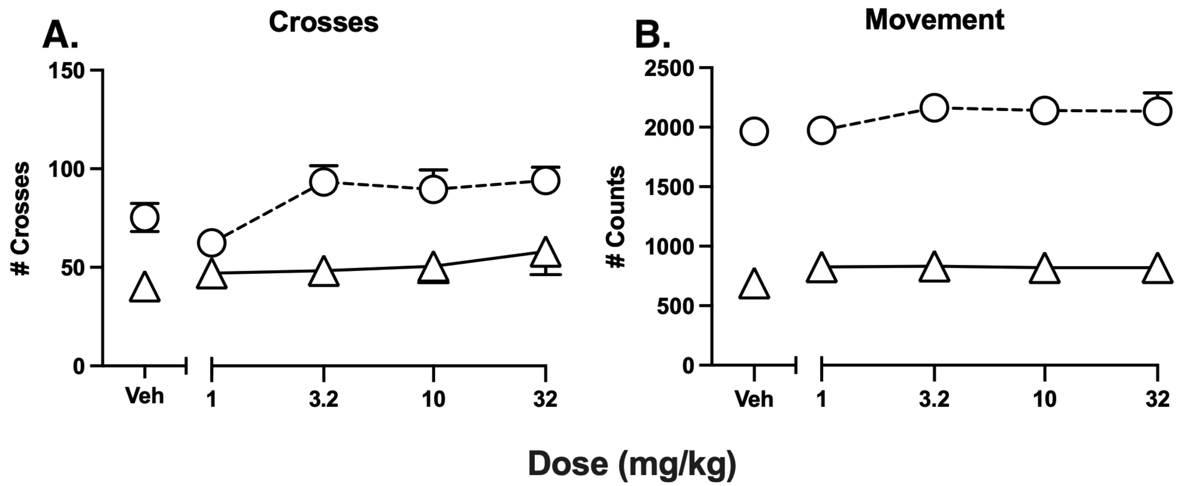


Figure 5.4 A - B Shows the effects a low-efficacy opioid. EG-1-230 effects alone and in the presence of 0.56% IP lactic acid in (A) crosses and (B) movement. Abscissa: Dose of opioid in mg/kg. For panel A the ordinate is number of crosses and for panel B the ordinate is number of counts.

Table 5.3: One-way ANOVA results for Figures 5.1 – 5.4.

Treatment	Crosses F statistic, p value	Movement F statistic, p value
Buprenorphine	F (4, 55) = 6.062; P=0.0004	F (4, 55) = 1.517; P=0.2099
Buprenorphine + LA	F (4, 55) = 7.170; P=0.0001	F (4, 55) = 8.166; P<0.0001
DC-01-128.1	F (4, 55) = 34.66; P<0.0001	F (4, 55) = 5.838; P=0.0005
DC-01-128.1 + LA	F (4, 55) = 1.064; P=0.3830	F (4, 55) = 8.213; P<0.0001
DC-01-0076.2	F (4, 55) = 2.720; P=0.0387	F (4, 55) = 1.279; P=0.2895
DC-01-0076.2 + LA	F (4, 54) = 2.268; P=0.0738	F (4, 54) = 25.58; P<0.0001
EWB-3-14	F (4, 55) = 11.39; P<0.0001	F (4, 55) = 3.797; P=0.0085
EWB-3-14 + LA	F (4, 55) = 5.249; P=0.0012	F (4, 55) = 13.67; P<0.0001
JL-02-0039	F (4, 55) = 7.815; P<0.0001	F (4, 55) = 1.151; P=0.3425
JL-02-0039 + LA	F (4, 55) = 3.588; P=0.0114	F (4, 55) = 4.976; P=0.0017
DC-01-0076.1	F (4, 55) = 1.981; P=0.1103	F (4, 55) = 2.221; P=0.0785
DC-01-0076.1 + LA	F (4, 55) = 3.423; P=0.0143	F (4, 55) = 6.223; P=0.0003
EG-1-203	F (4, 55) = 3.231; P=0.0188	F (4, 55) = 0.8794; P=0.4824
EG-1-203 + LA	F (4, 55) = 0.6505; P=0.6290	F (4, 55) = 0.4748; P=0.7540
EG-1-230	F (4, 55) = 0.8166; P=0.5201	F (4, 55) = 0.5051; P=0.7321
EG-1-230 + LA	F (4, 55) = 0.8099; P=0.5242	F (4, 55) = 0.5289; P=0.7149

Statistical analysis for all experiments were segregated by sex and are shown in **Tables 5.4 – 5.11**. There were no dose x sex interactions for any experiment; however, there was a main effect of sex in the following groups: DC-01-0076.2 + IP acid for movement, JL-02-0039 + IP acid for crosses, DC-01-0076.1 for movement, DC-01-0076.1 + IP acid for crosses, EG-1-203 for crosses, and EG-1-203 + IP acid for crosses.

Table 5.4: Two-way ANOVA results with power analysis for Figure 5.1 C – D [buprenorphine].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses Buprenorphine	Dose Main Effect	0.320	F (4, 50) = 5.89; P=0.0006	0.686	0.991	33
	Sex Main Effect	0.004	F (1, 50) = 0.19; P=0.6689	0.061	0.075	>100
	Dose x Sex Interaction	0.061	F (4, 50) = 0.81; P=0.5249	0.255	0.285	>100
Movement Buprenorphine	Dose Main Effect	0.113	F (4, 50) = 1.58; P=0.1934	0.356	0.534	100
	Sex Main Effect	0.020	F (1, 50) = 1.04; P=0.3139	0.144	0.191	>100
	Dose x Sex Interaction	0.113	F (4, 50) = 1.59; P=0.1921	0.356	0.536	100
Crosses Buprenorphine + LA	Dose Main Effect	0.369	F (4, 50) = 7.31; P=0.0001	0.765	0.993	28
	Sex Main Effect	0.023	F (1, 50) = 1.19; P=0.2788	0.155	0.218	>100
	Dose x Sex Interaction	0.089	F (4, 50) = 1.27; P=0.3118	0.313	0.423	>100
Movement Buprenorphine + LA	Dose Main Effect	0.398	F (4, 50) = 8.27; P<0.0001	0.813	0.999	26
	Sex Main Effect	0.000075	F (1, 50) = 0.0037; P=0.9512	0.009	0.050	>100
	Dose x Sex Interaction	0.102	F (4, 50) = 1.42; P=0.2403	0.337	0.486	>100

Table 5.5: Two-way ANOVA results with power analysis for Figure 5.2 A – B [DC-01-128.1].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses DC-01-128.1	Dose Main Effect	0.746	F (4, 50) = 36.65; P<0.0001	1.712	1	14
	Sex Main Effect	0.024	F (1, 50) = 1.22; P=0.2751	0.156	0.220	>100
	Dose x Sex Interaction	0.122	F (4, 50) = 1.74; P=0.1570	0.373	0.578	94
Movement DC-01-128.1	Dose Main Effect	0.308	F (4, 50) = 5.57; P=0.0009	0.667	0.987	34
	Sex Main Effect	0.031	F (1, 50) = 1.60; P=0.2117	0.179	0.274	>100
	Dose x Sex Interaction	0.017	F (4, 50) = 0.22; P=0.9288	0.131	0.102	>100
Crosses DC-01-128.1 + LA	Dose Main Effect	0.080	F (4, 50) = 1.09; P=0.3717	0.295	0.378	>100
	Sex Main Effect	0.005	F (1, 50) = 0.26; P=0.6109	0.072	0.085	>100
	Dose x Sex Interaction	0.108	F (4, 50) = 1.51; P=0.2124	0.348	0.514	>100
Movement DC-01-128.1 + LA	Dose Main Effect	0.395	F (4, 50) = 8.16; P<0.0001	0.808	0.999	26
	Sex Main Effect	0.009	F (1, 50) = 0.44; P=0.5088	0.094	0.100	>100
	Dose x Sex Interaction	0.077	F (4, 50) = 1.04; P=0.3952	0.289	0.362	>100

Table 5.6: Two-way ANOVA results with power analysis for Figure 5.2 C – D [DC-01-0076.2].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses DC-01-0076.2	Dose Main Effect	0.171	F (4, 50) = 2.57; P=0.0489	0.454	0.772	65
	Sex Main Effect	0.015	F (1, 50) = 0.76; P=0.3865	0.124	0.155	>100
	Dose x Sex Interaction	0.025	F (4, 50) = 0.32; P=0.8653	0.159	0.130	>100
Movement DC-01-0076.2	Dose Main Effect	0.089	F (4, 50) = 1.22; P=0.3142	0.312	0.421	>100
	Sex Main Effect	0.0003	F (1, 50) = 0.02; P=0.8925	0.019	0.052	>100
	Dose x Sex Interaction	0.047	F (4, 50) = 0.61; P=0.6561	0.221	0.220	>100
Crosses DDC-01-0076.2 + LA	Dose Main Effect	0.145	F (4, 49) = 2.08; P=0.0973	0.412	0.670	78
	Sex Main Effect	0.0001	F (1, 49) = 0.004; P=0.9444	0.010	0.051	>100
	Dose x Sex Interaction	0.0176	F (4, 49) = 0.22; P=0.9267	0.134	0.103	>100
Movement DC-01-0076.2 + LA	Dose Main Effect	0.691	F (4, 49) = 27.43; P<0.0001	1.496	1	15
	Sex Main Effect	0.098	F (1, 49) = 5.33; P=0.0252	0.330	0.699	77
	Dose x Sex Interaction	0.076	F (4, 49) = 1.01; P=0.4133	0.287	0.351	>100

Table 5.7: Two-way ANOVA results with power analysis for Figure 5.2 E – F [EWB-3-14].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses EWB-3-14	Dose Main Effect	0.477	F (4, 50) = 11.41; P<0.0001	0.955	0.999	21
	Sex Main Effect	0.036	F (1, 50) = 1.87; P=0.1775	0.193	0.312	>100
	Dose x Sex Interaction	0.061	F (4, 50) = 0.807; P=0.5267	0.254	0.284	>100
Movement EWB-3-14	Dose Main Effect	0.224	F (4, 50) = 3.59; P=0.0118	0.537	0.906	48
	Sex Main Effect	0.0001	F (1, 50) = 0.005; P=0.9421	0.010	0.051	>100
	Dose x Sex Interaction	0.041	F (4, 50) = 0.53; P=0.7130	0.206	0.195	>100
Crosses EWB-3-14 + LA	Dose Main Effect	0.291	F (4, 50) = 5.121; P=0.0015	0.640	0.979	36
	Sex Main Effect	0.043	F (1, 50) = 2.27; P=0.1381	0.213	0.367	>100
	Dose x Sex Interaction	0.027	F (4, 50) = 0.35; P=0.8456	0.166	0.138	>100
Movement EWB-3-14 + LA	Dose Main Effect	0.506	F (4, 50) = 12.78; P<0.0001	1.011	0.999	20
	Sex Main Effect	0.001	F (1, 50) = 0.07; P=0.7886	0.038	0.060	>100
	Dose x Sex Interaction	0.026	F (4, 50) = 0.34; P=0.8514	0.164	0.136	>100

Table 5.8: Two-way ANOVA results with power analysis for Figure 5.3 A – B [JL-02-0039].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses JL-02-0039	Dose Main Effect	0.367	F (4, 50) = 7.26; P=0.0001	0.762	0.998	28
	Sex Main Effect	0.00000045	F (1, 50) = 0.000002263; P=0.9962	0.00067	0.050	>100
	Dose x Sex Interaction	0.021	F (4, 50) = 0.27; P=0.8937	0.148	0.118	>100
Movement JL-02-0039	Dose Main Effect	0.081	F (4, 50) = 1.10; P=0.3661	0.297	0.382	>100
	Sex Main Effect	0.042	F (1, 50) = 2.20; P=0.1447	0.209	0.357	>100
	Dose x Sex Interaction	0.009	F (4, 50) = 0.11; P=0.9776	0.095	0.078	>100
Crosses JL-02-0039 + LA	Dose Main Effect	0.237	F (4, 50) = 3.88; P=0.0080	0.557	0.927	45
	Sex Main Effect	0.094	F (1, 50) = 5.18; P=0.0271	0.322	0.686	80
	Dose x Sex Interaction	0.079	F (4, 50) = 1.08; P=0.3788	0.293	0.374	>100
Movement JL-02-0039 + LA	Dose Main Effect	0.283	F (4, 50) = 4.94; P=0.0019	0.629	0.975	37
	Sex Main Effect	0.022	F (1, 50) = 1.13; P=0.2935	0.150	0.207	>100
	Dose x Sex Interaction	0.066	F (4, 50) = 0.88; P=0.4842	0.265	0.308	>100

Table 5.9: Two-way ANOVA results with power analysis for Figure 5.3 C – D [DC-01-0076.1].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses DC-01-0076.1	Dose Main Effect	0.138	F (4, 50) = 2.01; P=0.1076	0.401	0.651	82
	Sex Main Effect	0.053	F (1, 50) = 2.79; P=0.1013	0.236	0.434	>100
	Dose x Sex Interaction	0.057	F (4, 50) = 0.75; P=0.5646	0.244	0.264	>100
Movement DC-01-0076.1	Dose Main Effect	0.158	F (4, 50) = 2.35; P=0.0672	0.433	0.728	71
	Sex Main Effect	0.090	F (1, 50) = 4.97; P=0.0304	0.315	0.668	84
	Dose x Sex Interaction	0.059	F (4, 50) = 0.7771; P=0.5453	0.249	0.274	>100
Crosses DC-01-0076.1 + LA	Dose Main Effect	0.23537805	F (4, 50) = 3.848; P=0.0084	0.555	0.926	46
	Sex Main Effect	0.166	F (1, 50) = 9.97; P=0.0027	0.446	0.924	43
	Dose x Sex Interaction	0.036	F (4, 50) = 0.46; P=0.7615	0.193	0.174	>100
Movement DC-01-0076.1 + LA	Dose Main Effect	0.331	F (4, 50) = 6.18; P=0.0004	0.703	0.994	31
	Sex Main Effect	0.006	F (1, 50) = 0.31; P=0.5807	0.079	0.091	>100
	Dose x Sex Interaction	0.080	F (4, 50) = 1.09; P=0.3730	0.295	0.378	>100

Table 5.10: Two-way ANOVA results with power analysis for Figure 5.3 E – F [EG-1-203].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses EG-1-203	Dose Main Effect	0.205	F (4, 50) = 3.23; P=0.0197	0.508	0.869	53
	Sex Main Effect	0.083	F (1, 50) = 4.51; P=0.0387	0.300	0.626	92
	Dose x Sex Interaction	0.008	F (4, 50) = 0.10; P=0.9836	0.087	0.072	>100
Movement EG-1-203	Dose Main Effect	0.067	F (4, 50) = 0.907; P=0.4684	0.269	0.317	>100
	Sex Main Effect	0.001	F (1, 50) = 0.06; P=0.8096	0.034	0.058	>100
	Dose x Sex Interaction	0.115	F (4, 50) = 1.63; P=0.1809	0.361	0.549	99
Crosses EG-1-203 + LA	Dose Main Effect	0.056	F (4, 50) = 0.75; P=0.5639	0.245	0.265	>100
	Sex Main Effect	0.099	F (1, 50) = 5.51; P=0.0229	0.332	0.713	76
	Dose x Sex Interaction	0.134	F (4, 50) = 1.94; P=0.1188	0.394	0.633	84
Movement EG-1-203 + LA	Dose Main Effect	0.035	F (4, 50) = 0.45; P=0.7742	0.189	0.168	>100
	Sex Main Effect	0.0006	F (1, 50) = 0.03; P=0.8568	0.026	0.054	>100
	Dose x Sex Interaction	0.033	F (4, 50) = 0.43; P=0.7849	0.186	0.164	>100

Table 5.11: Two-way ANOVA results with power analysis for Figure 5.4 A – B. [EG-1-230].

Treatment	Dependent measure	Partial eta2	F statistic, p value	Cohen's Effect Size (Cohen's F)	Current Power	Sample Size: Power≥0.8
Crosses EG-1-230	Dose Main Effect	0.058	F (4, 50) = 0.77; P=0.5520	0.248	0.2707	>100
	Sex Main Effect	0.001	F (1, 50) = 0.06; P=0.8125	0.034	0.0575	>100
	Dose x Sex Interaction	0.031	F (4, 50) = 0.39; P=0.8123	0.177	0.1522	>100
Movement EG-1-230	Dose Main Effect	0.037	F (4, 50) = 0.48; P=0.7520	0.195	0.17774	>100
	Sex Main Effect	0.001	F (1, 50) = 0.07; P=0.7956	0.037	0.059	>100
	Dose x Sex Interaction	0.037	F (4, 50) = 0.48; P=0.7471	0.197	0.1798	>100
Crosses EG-1-230 + LA	Dose Main Effect	0.058	F (4, 50) = 0.77; P=0.5470	0.249	0.2733	>100
	Sex Main Effect	0.003	F (1, 50) = 0.14; P=0.7064	0.054	0.0691	>100
	Dose x Sex Interaction	0.047	F (4, 50) = 0.61; P=0.6548	0.223	0.2206	>100
Movement EG-1-230 + LA	Dose Main Effect	0.042	F (4, 50) = 0.55; P=0.7017	0.209	0.1995	>100
	Sex Main Effect	0.011	F (1, 50) = 0.55; P=0.4615	0.105	0.1254	>100
	Dose x Sex Interaction	0.113	F (4, 50) = 1.60; P=0.1902	0.357	0.5381	>100

5.4. Summary

After initial validation of a novel assay of pain-depressed behavior in mice, this study evaluated the effects of novel single-molecule opioids in the absence and presence of IP acid as an acute pain stimulus. There were four main findings. First, two different investigators tested the effects of different concentrations of IP acid and results indicated a concentration-dependent IP acid effect with no effect of investigator. Second, when the opioids were tested alone, their ability to alter crosses and movement was dependent on their efficacy. Third, when the opioids were tested in the presence of IP acid, alleviation of the pain-depressed behavior was also efficacy dependent, with optimal effects for the intermediate-efficacy opioids. Finally, sex effects were rare, and results suggest that when a sex effect was determined, it was a male-led effect.

Chapter Six

Expression of chronic pain-depressed behavior.

6.1. Introduction

Studies reported in **Chapters 4-5** in this dissertation used intraperitoneal injection of dilute lactic acid (IP acid) as a noxious stimulus. IP acid is useful as a noxious stimulus in preclinical pain studies because it is physiologically relevant, it produces robust and analgesic-reversible behavioral effects, it is easy and fast to deliver, and its intensity can be precisely manipulated by adjusting the dose. Additionally, it has a short duration of action of 1-2 hours, which allows sufficient time for many types of behavioral tests but is also short enough to permit repeated within-subject testing. However, most human pain states that warrant pharmacological treatment involve injury or disease and have longer durations of action. Specifically, pain states in humans are often categorized as "acute" or "chronic" depending on their duration of action. As introduced in **section 1.4.** of this dissertation, acute pain is defined as pain that is sudden and may be sharp or intense in sensation, usually has a root cause (e.g. injury, illness) and resolves within 6 months. For example, post-surgical pain includes both physical injury that directly activates nociceptors and inflammation that produces a sustained hypersensitivity of nociceptors. Chronic pain on the other hand, is defined as pain that lasts more than 3 months which extends beyond tissue healing time. For example, peripheral neuropathy is a result of damaged nociceptors that may have spontaneous activity and cause sensitization of secondary nociceptors. Chronic pain Epidemiological studies suggest that an average of 20.4% of the American population suffers from a chronic pain condition (Dahlhamer et

al., 2018; Yong et al., 2022) such as post-surgical pain that does not resolve, inflammatory arthritis (Lee, 2013), and neuropathy (Shiao and Lee-Kubli, 2018). While chronic pain is difficult enough to treat, high-impact chronic pain refers to chronic pain that is harsh enough to limit daily life or work-like activities, and an estimated 8% of the American population suffers from such high-impact chronic pain (Zelaya, 2020). These limitations of day-to-day function can be also referred to as functional impairment, and it is often a common reason why patients seek out professional help in hopes to restore behavior that is impaired back to normal (Cleeland and Ryan, 1994; Dworkin et al., 2005). Preclinically, efforts have been made to study and validate models of chronic pain that better reflect the disease model in human patients. Here we aim to evaluate pain-related behavioral depression produced by three preclinical pain models that vary in their mechanisms for producing pain states and the duration of pain-related behaviors they elicit (**Chapter 1; section 1.8).** As mentioned in the Introduction of this dissertation (**Chapter 1; section 1.5.2**), pain-depressed behaviors are studied as a means for better preclinical-to-clinical translational outcomes. The three different pain states studied here were (1) a local inflammatory model induced by complete Freund's Adjuvant (CFA) which is composed to heat-killed bacteria, (2) a post-surgical model induced by laparotomy, and (3) a mononeuropathic model induced by the spared nerve injury (SNI). We hypothesize that each of these pain models will produce sustained and robust depression of behavior, with longer depression of behavior produced by the SNI neuropathic pain model than by the CFA inflammatory pain model or laparotomy model of post-surgical pain.

6.2. Methods and materials

6.2.1. Subjects

Subjects were male and female ICR mice (Envigo, Frederick, MD) that were 6–8 weeks old upon arrival to the laboratory. Mice were segregated by sex and single-housed with corncob bedding (Envigo), a “nestlet” composed of pressed cotton (Ancare, Bellmore, NY), a cardboard tube for enrichment, and ad libitum access to food (Teklad LM-485 Mouse/Rat Diet; Envigo). Some males were split into smaller groups or isolated to minimize fighting. Cages were mounted in a RAIR HD Ventilated Rack (Laboratory Products, Seaford, DE) in a temperature-controlled room with a 12-hour light/dark cycle (lights on from 6:00 AM to 6:00 PM) in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed during the light phase of the daily light/dark cycle beginning 1 week after arrival at the laboratory. Animal-use protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and complied with the National Research Council Guide for the Care and Use of Laboratory Animals.

6.2.2. Chemicals

Complete Freund’s adjuvant (CFA) and sterile saline were purchased from Sigma Aldrich (St. Louis, MO).

6.2.3 Experimental Pain Manipulations

The primary goal of this study was to determine the duration and magnitude of pain-depressed behavior produced by experimental models of chronic pain. This was

accomplished by studying three different chronic pain models that vary in their mechanism (**Chapter 1; section 1.8**). Mice were randomly assigned to a treatment group (saline vs CFA) or surgical group (sham vs laparotomy or sham vs SNI). The experimenter was not blinded to treatment because data collection was automated by the Med Associates software. There were no exclusion criteria, and all data from all mice were included in final analysis.

6.2.4 Complete Freund's Adjuvant (CFA) procedure + paw width measurements.

Mice were briefly anesthetized via Isoflurane, and 30 µl of complete Freund's adjuvant (CFA) or saline was given via an intraplanar (Ipl) injection in the left hind-paw using a 27-gauge needle (Gould et al., 2016). To assess inflammation severity, paw-width was measured as described below.

6.2.5. Laparotomy procedure.

Surgeries were performed as described previously (Kendall et al., 2016; Oliver et al., 2018; Ulker et al., 2022). Briefly, mice were anesthetized via Isoflurane and abdominal hair was shaved at the surgical site using hair clippers (Amazon Inc, SweetLF, RFCD-3020) and disinfected with beta-iodine before surgery. A 1.0-1.5 cm vertical midline abdominal surgical incision was made through the skin and extended through the linea alba. To mimic visceral manipulation performed during various surgeries, a sterile cotton swab was inserted in the abdominal cavity and moved around for approximately 30 seconds. The abdominal layer and skin were closed using 5-0 nylon sutures, and the incision area was disinfected with beta-iodine. Once animals recovered from the anesthesia, they were placed back in their home cages, allowed to recover, and

monitored for any abnormalities. Sham surgical animals were anesthetized, abdominal hair was shaved at the abdominal site and disinfected with beta-iodine, but no surgical incisions were made. The average surgical time was 7-10 minutes per mouse.

6.2.6 Spared nerve injury (SNI) procedure.

Surgeries were performed as described perilously (Decosterd and Woolf, 2000; Shields et al., 2003). Briefly, mice were anesthetized via isoflurane, and the skin on the lateral surface of the left thigh was shaved followed by topical application of iodine solution. A single small incision was made at the mid-thigh level of the lateral surface of the thigh with fine scissors using the left knee as a landmark. A blunt dissection was performed through the bicep femoris muscle to expose the sciatic nerve and its three terminal branches (sural, tibial, and common peroneal). The common peroneal and the tibial nerves were carefully isolated and tightly ligated using a 6-0 silk suture, and sectioned distal to the ligation, removing ~2-4 mm of the distal nerve. The sural nerve was spared. The overlying muscle was closed using a 6-0 vicryl suture, the skin was closed with a 6-0 silk suture, and local antiseptic cream was applied to the wound. Once animals recovered from the anesthesia, they were placed back in their home cages and monitored for any abnormalities. In sham mice, the sciatic nerve and its branches were exposed, but without any manipulation of nerves. Muscle and skin were closed in layers in the same way as SNI group. The average surgical time was 7-10 minutes per mouse.

6.2.7. Paw mechanical sensitivity procedure.

To assess mechanical sensitivity to paw stimulation with von Frey filaments, the up-down method was performed as described previously with some modifications

(González-Cano et al., 2020; Morgan et al., 2022). Briefly, animals were placed in clear plexiglass enclosures on a mesh-like stand with ¼" waffle sized holes (IITC Life Science). Up to 12 mice were tested at once and mice were acclimated in the experimental room and enclosures for one hour before testing. After acclimation, calibrated von Frey filaments were used (Stoelting Co) (Chaplan et al., 1994) and applied to the mid-plantar surface of the left paw beginning with the mid-rage filament (0.6 g) until bent for a duration of at least 3 seconds or a response was observed, with a response counted as paw withdraw with licking or shaking. If a response was observed, a smaller filament was used (0.4g) and if a response was not observed the next largest filament was used (1.0 g). Measurements were determined after the behavioral session concluded.

6.2.8 Dependent variables

The behavioral variables measured were (1) "Crosses" defined as the number of crosses between the compartments, which required mice to rear and surmount the vertical barrier in the doorway, and (2) "Movement" defined as the total number of beam breaks in each individual compartment, which required only horizontal locomotor activity. In the CFA group, behavioral measurements were determined in 15-minute sessions with barrier height measuring 1 inch (2.54 cm). To assess paw inflammation, paw-width measurements were determined with calipers to the nearest 0.01 mm after the behavioral session concluded. Behavioral and paw-width measurements were done post-injection at 6-, 24-, and 72-hours. In the laparotomy group, behavioral measurements were determined in 60-minutes with 15-minute bin breakdowns to compliment the other behavioral procedures. The barrier height measured 1 inch (2.54 cm) and time to test post-surgery was 2-, 6-, 24-, and 72-hours. In the SNI group, behavioral measurements

were determined in 15-minute sessions with the barrier height measuring 1.5 inches (3.81 cm). To assess mechanical sensitivity in the paw, von Frey monofilaments were used after the behavioral session concluded. Time to test post-surgery was 14-, 28-, 56-, and 105-days.

6.2.9. Data Analysis.

The present study included both females and males in accordance with National Institutes of Health Guidelines (Miller et al., 2017), but it was not intended a priori to detect sex differences. Accordingly, for initial data analysis, males and females were pooled and analyzed by a two-way ANOVA with treatment and time as factors. A significant main effect of treatment or treatment × time interaction was followed by a Holm-Sidak post-hoc test. To provide a secondary assessment sex effect, data were segregated by sex and analyzed via a three-way ANOVA with sex, time, and treatment as factors. A significant main effect of sex or interaction involving sex was followed by a Holm-Sidak post-hoc test. The criterion for significance was $P < 0.05$. This data analysis was performed using GraphPad Prism 9.5 (La Jolla, CA)

6.3. Results

Figure 6.1 shows that CFA produced significant paw inflammation that persisted for at least three days, and that CFA had a main effect on movement but not crosses. This figure also illustrates the statistical analysis for all subsequent experiments. Thus, **Figure 6.1.A** shows that CFA significantly increased paw inflammation as there was a main effect of treatment [$F(1, 66) = 197.4$; $P<0.0001$], a main effect of time [$F(2, 66) = 40.39$; $P<0.0001$], and a significant time x treatment interaction [$F(2, 66) = 12.96$;

P<0.0001]. Post-hoc analysis indicated that there was a difference between saline and CFA animals at 6-hours, 24-hours, and 72-hours post-injection. **Figure 6.1.B** shows that CFA did not alter the number of crosses as there was no main effect of treatment [F (1, 66) = 0.3560; P=0.5527], no main effect of time [F (2, 66) = 3.338; P=0.0416], and no significant time x treatment interaction [F (2, 66) = 0.6801; P=0.5101]. **Figure 6.1.C** shows that CFA did alter movement counts as there was a main effect of group [F (1, 66) = 4.622; P=0.0352], a main effect of time [F (2, 66) = 5.427; P=0.0066], but no significant time x group interaction [F (2, 66) = 2.027; P=0.1399].

To determine if there was an effect of sex, data from males and females were segregated and three-way ANOVAs were conducted with sex as a variable. Paw-width data shows that there was a main effect of sex [F (1, 60) = 7.910; P=0.0066]. There was no significant time x sex interaction [F (2, 60) = 2.687; P=0.0763], treatment x sex [F (1, 60) = 0.07280; P=0.7882], or time x treatment x sex [F (2, 60) = 0.3668; P=0.6945]. Results for crosses show that there was no main effect of sex [F (1, 60) = 1.894; P=0.1739], no significant time x sex interaction [F (2, 60) = 1.796; P=0.1748], treatment x sex [F (1, 60) = 1.220; P=0.2737], or time x treatment x sex [F (2, 60) = 0.9646; P=0.3870]. Results for movement indicate that there was no main effect of sex [F (1, 60) = 3.216; P=0.0780], no significant time x sex interaction [F (2, 60) = 1.268; P=0.2888], treatment x sex [F (1, 60) = 1.572; P=0.2147], or time x treatment x sex [F (2, 60) = 0.6347; P=0.5336].

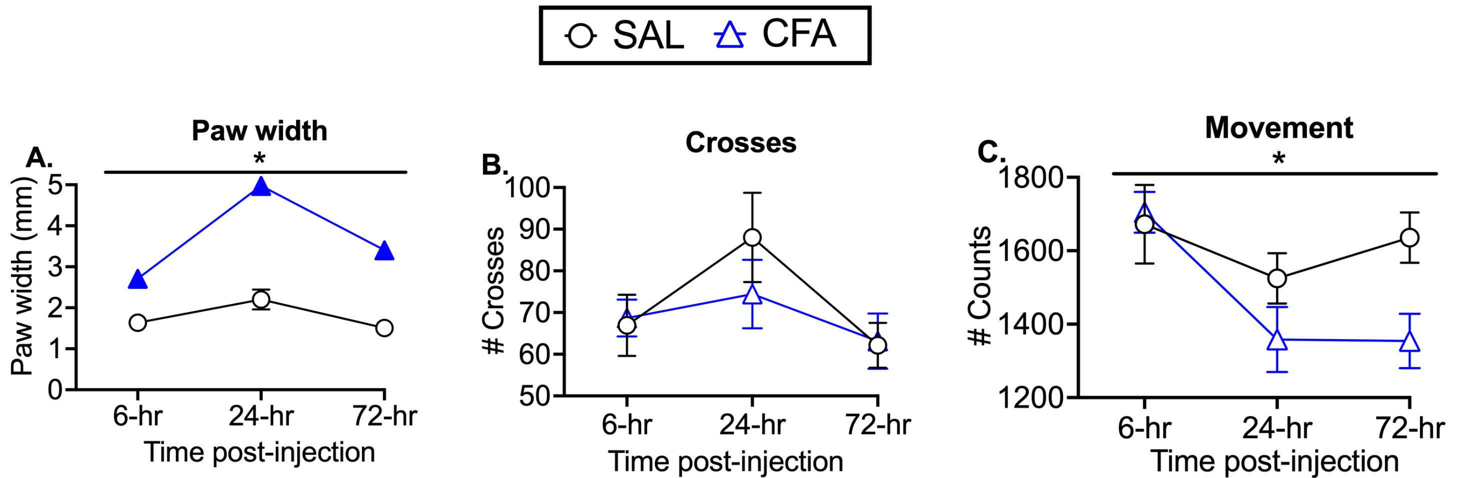


Figure 6.1: Effects of Intraplanar CFA. (A) Effects of CFA on paw-width. Ordinate: Paw width (mm). (B) Effects of CFA on crosses. Ordinate: number of crosses. (C). Effects of CFA on movement. Ordinate: number of counts. Abscissa for all panels: Time to test post-injection at 6-hr, 24-hr, and 72-hr. Asterisk in panels A and C shows a main effect of group *p<0.01. Filled points in panel A indicate a significant difference from the "SAL" group p<0.05.

Figure 6.2 shows that the laparotomy model of post-surgical pain had an overall effect to alter crosses and movement. **Figure 6.2. A - B** shows data for a 15-minute behavioral session (to compliment the other chronic pain models), and **Figure 6.2. C - D** shows data for a 60-minute behavioral session. Thus, **Figure 6.2.A** shows that laparotomy did not alter the numbers of crosses as there was no main effect of treatment [F (1, 88) = 2.338; P=0.1299], no main effect of time [F (3, 88) = 1.205; P=0.3126], and no significant time x treatment interaction [F (3, 88) = 1.005; P=0.3945]. **Figure 6.2.B** shows that laparotomy did alter movement counts as there was a main effect of treatment [F (1, 88) = 6.817; P=0.0106], but no main effect of time [F (3, 88) = 1.073; P=0.3648], and no significant time x treatment interaction [F (3, 88) = 2.643; P=0.0542]. **Figure 6.2.C** shows that laparotomy did alter the number of crosses as there was a main effect of treatment [F (1, 88) = 6.944; P=0.0099], but no main effect of time [F (3, 88) = 1.120; P=0.3455], and no significant time x treatment interaction [F (3, 88) = 1.524; P=0.2140]. **Figure 6.2.D** shows that laparotomy did alter movement counts as there was a main effect of treatment [F (1, 88) = 27.19; P<0.0001], a main effect of time [F (3, 88) = 4.546; P=0.0052], and no significant time x treatment interaction [F (3, 88) = 1.877; P=0.1392].

To determine if there was an effect of sex, data from males and females were segregated and three-way ANOVAs were conducted with sex as a variable. For the 15-minute session, there was no main effect of sex for crosses [F (1, 80) = 0.4567; P=0.5011], or significant time x sex interaction [F (3, 80) = 2.420; P=0.0721], treatment x sex [F (1, 80) = 1.300; P=0.2575], or time x treatment x sex [F (3, 80) = 0.5580; P=0.6443]. For movement, there was no significant sex effect [F (1, 80) = 1.207; P=0.2753], or significant time x sex interaction [F (3, 80) = 0.9276; P=0.4314], treatment x sex [F (1, 80)

= 0.1856; P=0.6678], or time x treatment x sex [F (3, 80) = 0.8589; P=0.4660]. For the 60-minute session, there was no main effect of sex for crosses [F (1, 80) = 0.005175; P=0.9428], a significant time x sex interaction [F (3, 80) = 2.830; P=0.0436], but no significant treatment x sex [F (1, 80) = 0.6977; P=0.4061], or time x treatment x sex [F (3, 80) = 0.3444; P=0.7933] interaction. For movement, there was no main effect of sex, [F (1, 80) = 2.100; P=0.1512], or significant time x sex interaction [F (3, 80) = 2.016; P=0.1183], treatment x sex [F (1, 80) = 1.141; P=0.2887], or time x treatment x sex [F (3, 80) = 1.707; P=0.1721].

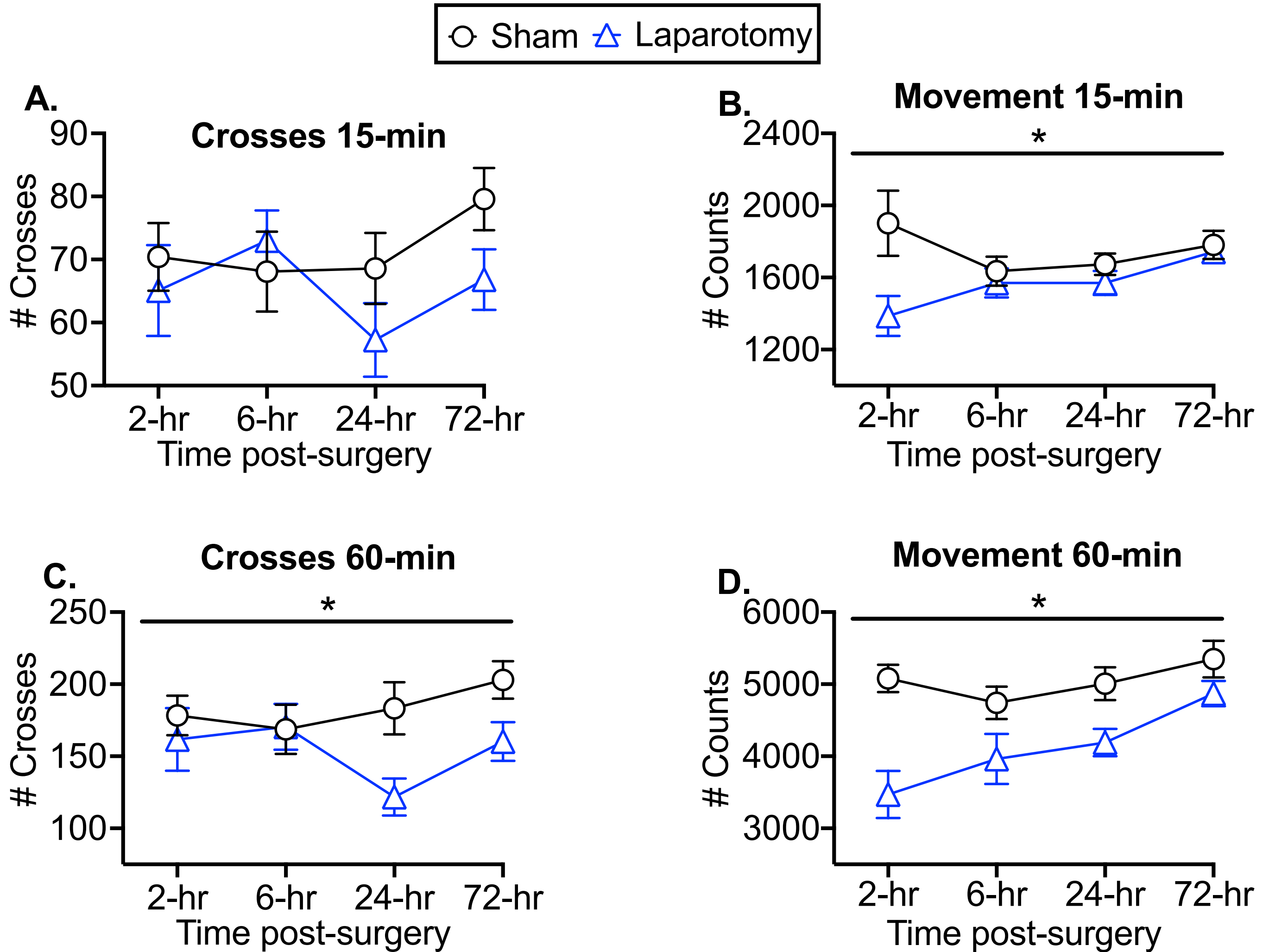


Figure 6.2: Effects of laparotomy. Panels A – B show data for the 15-minute behavioral sessions, and Panels C - D shows data for the 60-minute behavioral sessions. (A and C) Effects of laparotomy on crosses. Ordinate: number of crosses. (B and D). Effects of laparotomy on movement. Ordinate: number of counts. Abscissa for all panels: Time to test post-surgery at 2-hr, 6-hr, 24-hr, and 72-hr. Asterisk in panels B, C and D show a main effect of group *p<0.01.

Figure 6.3 shows that SNI produced sustained mechanical hypersensitivity and that SNI had a greater effect on movement but not crosses. Thus, **Figure 6.3.A** shows that SNI had greater mechanical hypersensitivity as there was a main effect of treatment [F (1, 88) = 153.3; P<0.0001], a main effect of time [F (3, 88) = 3.499; P=0.0188], but no significant time x treatment interaction [F (3, 88) = 0.8241; P=0.4840]. **Figure 6.3.B** shows that SNI did not alter the number of crosses as there was no main effect of treatment [F (1, 88) = 0.7960; P=0.3747], however there was a main effect of time [F (3, 88) = 3.360; P=0.0223] but not a significant time x treatment interaction [F (3, 88) = 0.3525; P=0.7874]. **Figure 6.3.C** shows that SNI did alter movement counts as there was a main effect of treatment [F (1, 22) = 14.25; P=0.0010], no main effect of time [F (2.727, 59.99) = 0.8996; P=0.4389], and no significant time x treatment interaction [F (3, 66) = 2.037; P=0.1172].

To determine if there was an effect of sex, data from males and females were segregated and three-way ANOVAs were conducted with sex as a variable**.** There was no main effect of sex in mechanical hypersensitivity [F (1, 20) = 4.234; P=0.0529], or significant day x sex interaction [F (3, 60) = 0.5823; P=0.6289], treatment x sex [F (1, 20) = 0.3288; P=0.572], or day x treatment x sex [F (3, 60) = 0.3369; P=0.7987]. There was no main effect for crosses [F (1, 20) = 0.9974; P=0.3299], or significant day x sex interaction [F (3, 60) = 0.5059; P=0.6797], treatment x sex [F (1, 20) = 0.2926; P=0.5945], or day x treatment x sex interaction [F (3, 60) = 1.082; P=0.3636]. There was no main effect of sex for movement [F (1, 20) = 1.352; P=0.2586], or significant day x sex interaction [F (3, 60) = 0.4865; P=0.6930], treatment x sex [F (1, 20) = 0.04663; P=0.8312], or day x treatment x sex [F (3, 60) = 0.7880; P=0.5053].

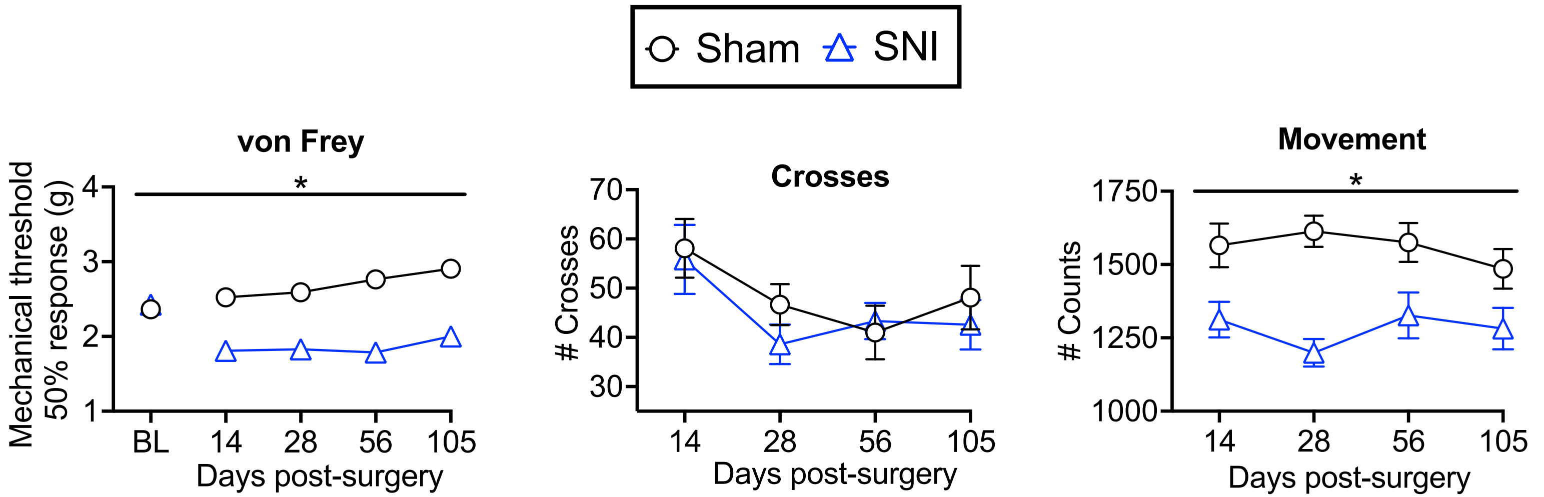


Figure 6.3: Effects of spared nerve injury (SNI). (A) Effects of SNI on mechanical sensitivity. Ordinate: mechanical threshold in g. (B) Effects of SNI on crosses. Ordinate: number of crosses. (C). Effects of SNI on movement. Ordinate: number of counts. Abscissa for all panels: Time to test at 14-, 28-, 56-, 105- days post-surgery. Asterisk in panels A and D show a main effect of group *p<0.01.

6.4. Summary.

This study evaluated the effects of three different experimental chronic pain models that varied in their mechanism and duration in an assay of pain-depressed behavior. There were four main findings. First, while CFA produced sustained paw inflammation, the behavioral effects were weak and only movement was significantly affected. Second, laparotomy showed greater behavioral effects by decreasing both crosses and movement. Third, while SNI produced sustained mechanical hypersensitivity, SNI only had an effect on overall movement but not crosses. Finally, these studies provide no evidence for sex differences as none were observed in both behavioral endpoints across groups.

Chapter Seven

Discussion

7.1. General Summary

This project focused on investigating the role of MOR efficacy in treatment of pain-depressed behavior. To investigate the role of MOR efficacy, three classes of opioid ligands were studied as listed below. Furthermore, this project was split into three different parts. Part I (**Chapters 2-3**) focused on investigating MOR ligand effects on horizontal locomotor activity in the absence of pain (as a “pain-independent” behavior). Part II (**Chapters 4-5**) focused on investigating a subset of the MOR ligands in the presence of an acute pain stimulus in two different assays of pain-depressed behavior. Part III (**Chapter 6**) focused on the expression of chronic pain in an assay of pain-depressed behavior.

1) Clinically available single-molecule opioids (listed from highest to lowest MOR efficacy)
    - Methadone
    - Fentanyl
    - Morphine
    - Hydrocodone
    - Buprenorphine
    - Nalbuphine
    - Naltrexone

2) Fentanyl/naltrexone (FENT/NTX) mixtures (listed from highest to lowest MOR efficacy)

- FENT/NTX 100:1
- FENT/NTX 56:1
- FENT/NTX 32:1
- FENT/NTX 10:1
- FENT/NTX 3.2:1
- FENT/NTX 1:1

3) Novel single-molecule opioids (listed from highest to lowest MOR efficacy)

- DC-01-128.1
- DC-01-76.2
- EWB-3-14
- JL-02-0039
- NAQ
- DC-01-76.1
- EG-1-203
- EG-1-230

7.2. Part I - Efficacy Dependence of MOR Agonist-Induced Hyperactivity in Mice.

Chapters 2 and **3** investigated opioid effects on locomotor activity in a rectangular open-field chamber during 60-min behavioral sessions. The results in **Chapters 2** and **3** agree with pervious findings in that MOR agonists produce locomotor activation in several strains of mice, including ICR mice (Bailey et al., 2010; Brase et al., 1977; Rethy et al.,

1971; Szumlinski et al., 2020). This hyperactivity is expressed as continuous, unidirectional, and thigmotactic rotation around the perimeter of available space with reduced vertical activity (i.e., rearing, climbing) (Marcais-Collado et al., 1983; Michael-Titus et al., 1989; Mickley et al., 1989), and it can be viewed as a sign of adverse MOR-agonist-induced motor disruption relative to other effects, such as antinociception, associated with therapeutic benefit. The present study expands on these previous findings in its explicit examination of MOR efficacy as a determinant of MOR agonist-induced hyperactivity.

Most single-molecule clinically available opioids and fentanyl/naltrexone mixtures produced dose dependent increases in locomotion, and peak levels of activity across different drugs and mixtures were associated with peak levels of MOR-coupled G-protein signaling as measured by in vitro assays of ligand-stimulated GTPɣS binding in CHO cells expressing cloned MORs. This relationship was well described by a linear function up to a point, suggesting that MOR agonist-induced locomotor activation is mediated by MOR-coupled G-protein signaling; however, drugs or mixtures that exceeded an in vitro Emax value of ~50% of the reference agonist DAMGO all produced similar Emax values for locomotor activation. These findings suggest that biologic or procedural constraints impose a ceiling on maximal locomotor activation by high-efficacy MOR agonists. Conversely, no significant locomotor activation was produced by the low-efficacy MOR agonist NAQ or by the low-proportion 3.2:1 fentanyl/naltrexone mixture, both of which produce low but detectable levels of ligand-stimulated GTPɣS binding (Selley et al., 2021; Yuan et al., 2013).

Taken together, these results show that MOR agonist-induced hyperactivity in mice is efficacy dependent, with graded E_{max} values within a range of low- to intermediate-efficacy agonists and a plateau of peak hyperactivity for high-efficacy agonists. Additionally, these results indicate that in vivo hyperactivity had a slightly higher efficacy threshold to detect agonist activity, a substantially lower ceiling, and a lower overall efficacy requirement than in vitro stimulation of GTPɣS binding for detection of MOR agonist effects. This study examined efficacy dependence of MOR agonist induced hyperactivity in mice using both single-molecule opioids and fentanyl/naltrexone mixtures. Results with the single-molecule opioids were suggestive of efficacy dependence, but the low-efficacy agonists nalbuphine and NAQ in this series have relatively low MOR selectivity (Pick et al., 1992; Yuan et al., 2011), and nalbuphine in particular produces agonist effects mediated by kappa opioid receptors (KOR) in mice (Narver, 2015; Patrick et al., 1999). Because KOR agonists decrease locomotor activity in mice (Gwynn and Domino, 1984; Kuzmin et al., 2000), it is possible that low locomotor activity with these drugs in general and nalbuphine in particular resulted from low selectivity for MOR versus KOR rather than from low MOR efficacy. However, fentanyl/naltrexone mixtures with low fentanyl proportions also produced low peak levels of hyperactivity. With the mixtures, all agonist effects are produced by the highly MOR-selective opioid fentanyl, and net efficacy is controlled by the inclusion of naltrexone to block MORs and limit the maximal number of receptors that can be occupied by fentanyl. Moreover, linear regression indicated that the magnitude of GTPɣS binding associated with an intermediate level of hyperactivity (50% of the Emax for fentanyl alone) was the same for single-molecule opioids and fentanyl/naltrexone mixtures. This suggests that low MOR efficacy is sufficient to explain

the low levels of hyperactivity produced by nalbuphine and NAQ in this study, although any additional KOR agonist effects may also have contributed. Overall, the inclusion of data with fentanyl/naltrexone mixtures strengthens the conclusion of efficacy dependence for MOR agonist-induced hyperactivity in mice.

Chapter 3 results agree with **Chapter 2** results to show that the MOR agonist-induced hyperactivity in mice is efficacy dependent. The main difference between Chapter 2 and 3 is that in Chapter 3, the compounds of interest were novel single-molecule opioids with graded efficacy and greater MOR selectivity (Chambers et al., 2022; Gutman et al., 2020, Lutz et al., 2023; *In Press*, Tom Prinsenzano; personal communication) . Results indicate that low-efficacy MOR agonists produced graded E_{max} effects until no effect was achieved. Two effects were observed with the high-efficacy MOR ligands (1) an Emax plateau of mouse hyperlocomotion, and (2) potency differences for the compounds to achieve this hyperactivity effect. To better understand if MORs were necessary to generate the effects produced by the novel single-molecule opioids, two types of antagonism studies were conducted. First, the opioids with detectable hyperactivity (tianeptine, DC-01-128.1, DC-1-76.2, EWB-3-14, JL-2-39, and DC-1-76.1) were tested after the administration of the antagonist naltrexone, and results indicated that naltrexone was effective to antagonize the hyperactivity produced by these single-molecule opioids. Second, because the two lowest-efficacy opioids (EG-1-203, EG-1-230) produced little-to no hyperactivity, they were tested as a pre-treatment to the high-efficacy MOR agonist morphine. Results indicated that the locomotor activating effects of morphine were blocked by increasing doses of the low-efficacy opioids. Results from these studies suggest a MOR selective mediated effect.

7.3. Part I - Efficacy Requirements for MOR Agonist-Induced Hyperactivity in Mice Relative to Other in Vivo Effects.

Chapter 2 results show the determination of dose-effect curves and Emax values for a range of fixed-proportion fentanyl/naltrexone mixtures, and these data provide a strategy to quantify efficacy requirements across opioid endpoints as the EP50 value, or the "effective proportion" of fentanyl in a fentanyl/naltrexone mixture required to produce an Emax equal to 50% of the fentanyl-alone Emax (Cornelissen et al., 2018; Schwienteck et al., 2019; Selley et al., 2021). For example, evidence cited above indicates that MOR agonist induced hyperactivity in mice has a lower efficacy requirement than ligand-stimulated GTPɣS binding in MOR CHO cells, and this conclusion is further supported and quantified by reference to the EP50 values, with the EP50 (95%CL) being significantly lower for hyperactivity in mice than for GTPɣS binding in MOR CHO cells. Two other general conclusions are suggested by a comparison of the present results with our previously published results (Cornelissen et al., 2018; Schwienteck et al., 2019; Selley et al., 2021). First, the EP50 for hyperactivity in mice was high relative to other in vivo behavioral endpoints in mice, rats, and monkeys, and in particular, was significantly higher than the EP50 from an assay of thermal nociception in mice. Insofar as opioid antinociception is related to a therapeutic opioid effect (analgesia) whereas hyperactivity is related to an adverse effect (motor disruption), these results provide evidence for the potential of low-efficacy MOR agonists to produce analgesic effects without producing at least some degree of motor impairment. It should be noted that the EP50 for hyperactivity in mice was not significantly lower than that for thermal antinociception in rats or monkeys, suggesting that the window of opportunity here is narrow; nonetheless, these findings agree with other evidence to suggest that low-efficacy opioids can produce thermal

antinociception without hyperactivity in mice (Varshneya et al., 2021, 2019). Second, the EP50 for hyperactivity in mice was also significantly higher than for a fentanyl discrimination assay in rats. Drug discrimination procedures model drug-induced subjective effects that may contribute to abuse potential, and as such, this finding suggests that abuse-related of MOR agonist effects have very low efficacy requirements. The fentanyl/naltrexone-mixture approach has not yet been applied to other endpoints of abuse related opioid effects; however, evidence using other approaches to assess efficacy requirements (e.g., comparing low- and high-efficacy agonists or evaluating abuse-related effects after MOR downregulation with irreversible antagonists or genetic receptor knockdown) has also suggested that abuse-related MOR effects have relatively low efficacy requirements (Negus and Moerke, 2019; Sora et al., 2001; Zernig et al., 1997). Thus, although both hyperactivity in mice and rewarding/reinforcing effects of opioids in multiple species all appear to be mediated at least in part by mesolimbic dopamine signaling as a common neural substrate, it appears that lower MOR efficacy is required for behavioral reward/reinforcement processes than for unconditioned hyperactivity. One implication of these findings is that low-efficacy MOR agonists may produce little or no evidence of hyperactivity in mice but nonetheless produce rewarding/reinforcing effects sufficient to underlie abuse potential.

7.4. Part II – Climbing as an assay of pain-depressed behavior.

In **Chapter 4**, climbing behavior was studied because it is an ethologically important component of locomotor activity for mice living in the wild, but it is rarely studied in the laboratory, and the impact of pain states on climbing behavior is unknown. This study assessed climbing in vertically oriented cylinders lined with wire mesh on the walls

and lid, and under baseline conditions, mice engaged in climbing for ~40% of the 10-min behavioral sessions. A few previous studies have also used vertically oriented compartments with scalable walls to assess climbing (Deacon and Rawlins, 2005; Layne, 1970; Marcais-Collado et al., 1983; Mori et al., 2003; Protais et al., 1976). For example, one series of studies used a vertically oriented cylinder lined with horizontal bars similar to our apparatus, and in agreement with our study, climbing scores under baseline conditions were approximately 40% of the maximum possible score (Marcais-Collado et al., 1983). This study built on these earlier studies in four ways. First, we measured time climbing as a continuous ratio variable rather than assigning ordinal scores for intermittently observed climbing behavior. This increased quantitative precision, justified the use of parametric statistics for analysis, and avoided conflation of rearing and climbing behaviors. Second, this study videotaped test sessions for later scoring to avoid having an investigator in the room as an extraneous variable (Sorge et al., 2014). The use of videotapes also facilitated parallel scoring by multiple observers to enable demonstration of high inter-rater reliability scores. Third, this study established stability of climbing both within individual mice during repeated testing and between different groups of mice tested over a period of months. The stability of climbing across days with individual mice justified subsequent within-subject experimental designs. The stability of climbing across multiple groups of mice treated 20-min before testing with SC saline (as a control for drug-alone studies) increased confidence that changes in climbing reflected drug effects rather than other extraneous factors that might vary across cohorts and time (note that decreases in climbing produced by 10-min pretreatment with IP water are discussed below). Lastly, our study included both male and female mice to assess the influence of sex as a biological

variable (Diester et al., 2019), and sex differences were small or absent throughout the study.

Results of this study agree with previous findings that IP injection of dilute acid can serve as an acute visceral noxious stimulus to produce a concentration-dependent decrease in a range of mouse and/or rat behaviors that include feeding (Kwilasz and Negus, 2012; Stevenson et al., 2006), horizontal locomotor activity (Stevenson et al., 2009), wheel running (Miller et al., 2011), nesting (Diester et al., 2021; Negus et al., 2015), and positively reinforced operant behavior (Baldwin et al., 2022; Brust et al., 2016; Carmo et al., 2009). In the present study, 10-min pretreatment with IP water (as the vehicle control for IP acid) significantly decreased climbing relative to 20-min pretreatment with SC saline alone. In addition, a follow-up pilot study found that 10-min pretreatment with IP saline did not decrease climbing (data not shown). These findings suggest that the hypotonic water solution was sufficient to produce some behavioral disruption; nonetheless, IP acid was still effective to produce a further concentration-dependent decrease in climbing. Taken together, these instances of IP acid-induced behavioral depression can be interpreted as evidence of “pain” because (a) acid injection can produce the subjective state of pain in humans (Laura et al., 2008), (b) acid injection in humans or laboratory animals can model tissue acidosis associated with many injury- and inflammation-associated pain states (Reeh and Steen, 1996), and (c) IP acid effects in laboratory animal studies cited above and in the present study were blocked by a clinically effective NSAID analgesic such as ketoprofen but not by a clinically ineffective negative control. To our knowledge, this is the first study to use a vertically oriented test environment with scalable walls to assess pain-related depression of climbing in mice;

however, in agreement with the present results, a wide range of experimental pain models has been found to depress a potentially related behavior called “cage-lid hanging” in mice (Falk et al., 2017; Roemers et al., 2019; Zhang et al., 2020). Cage-lid hanging is assessed in horizontally oriented home-cage environments with flat floors, unscalable plastic walls, and a wire lid (Pitzer et al., 2016; Zhang et al., 2020), and hanging behavior occurs when mice rear or jump to the wire lid and hang from it. Moreover, as in the present study, pain-related depression of cage-lid hanging was blocked by ketoprofen but not by a centrally acting kappa opioid receptor agonist as a negative control. Overall, these results support the use of mouse climbing behavior as an endpoint for studies of pain-related behavioral depression and its pharmacological modulation by candidate analgesics.

This study showed that single molecule opioids and fentanyl/naltrexone mixtures administered alone decreased climbing behavior in an efficacy- and dose-dependent manner in ICR mice. This agrees with previous work (Marcais-Collado et al., 1983), which showed that opioids potently decreased climbing behavior in an apparatus similar to the one used here. The present study builds on these previous findings by demonstrating that climbing is highly sensitive to disruption by MOR agonists and has a very low MOR efficacy requirement. Specifically, previous work in our lab has used fixed-proportion fentanyl/naltrexone mixtures as a strategy to quantify the efficacy requirements for a wide range of MOR agonist-induced behavioral endpoints in mice, rats, and rhesus monkeys (Cornelissen et al., 2018; Santos et al., 2022; Schwienteck et al., 2019; Selley et al., 2021). Application of this approach in the present study revealed that climbing in mice is the most sensitive behavioral effect we have evaluated in any species. For example, 10-

fold lower proportions of fentanyl in the fentanyl/naltrexone mixtures are sufficient to decrease climbing than to stimulate horizonal locomotion in mice, indicating that very low levels of MOR stimulation are necessary to depress climbing behavior. Climbing by mice can also be altered by some other classes of drugs, such as dopamine receptor agonists and antagonists (Costall et al., 1982; Kim et al., 1996; Marcais et al., 1978; Protais et al., 1976), but the relative sensitivity of climbing as a behavioral endpoint for drugs from other pharmacological classes has not been extensively evaluated. One implication of the present results is that depression of climbing is an especially sensitive endpoint for detection of behavioral impairment produced by MOR ligands, and this endpoint could be useful in characterizing the overall safety profile of MOR ligands or other drugs.

MOR agonists are widely used clinically as analgesics, but in contrast to the clinically effective NSAID analgesic ketoprofen, none of the single-molecule MOR agonists or fentanyl/naltrexone mixtures was effective to alleviate IP acid-induced depression of climbing. This finding likely reflects the high sensitivity of climbing to disruption by administration of the opioids alone. In any assay of pain-depressed behavior, drug effectiveness to alleviate pain-related behavioral depression will depend on an integration of at least two effects: (1) analgesic drug effects that reduce sensitivity to the noxious stimulus and will thereby tend to increase expression of the depressed behavior, and (2) direct effects of the drug on motor function that may impair behavior and tend to exacerbate behavioral depression and obscure analgesic effects (Baldwin et al., 2022). In the case of the NSAID ketoprofen, there was no effect on climbing when ketoprofen was administered alone, and this enabled unobstructed expression of analgesic blockade of the IP acid-induced depression of climbing. The MOR agonists and

fentanyl/naltrexone mixtures, by contrast, were both potent and effective to disrupt climbing when administered alone. As result, any blockade of IP acid effects produced by analgesic doses of these opioids was likely obscured by their direct disruption of climbing, and lower doses that did not disrupt climbing were also not sufficient to block IP acid effects.

7.5. Part II – Locomotor + barrier as an assay of pain-depressed behavior.

Chapter 4 results determined that climbing is an assay that is too sensitive to MOR-induced disruption to be able to detect an alleviation of the pain-depressed behavior. Because of this, we sought to validate a novel assay as described in **Chapter 5**, the "locomotor + barrier" assay, which uses a more complex locomotor environment to assess a combination of horizontal and vertical activity. Specifically, for the locomotor + barrier assay, the chamber consisted of two compartments separated by a door occluded by a wire-mesh barrier. To cross between the compartments, mice had to rear/climb over the barrier as a type of vertical activity, whereas locomotor activity within each compartment provided a measure of horizontal activity. Initial parametric validation steps of the novel assay determined the following. First, the locomotor + barrier assay was sensitive to increasing concentrations of IP acid, which was used as an acute pain stimulus as described in detail above in **section 7.4**. Second, the positive control NSAID ketoprofen was effective at blocking the pain-depressed behavior. Third, the negative controls diazepam, U69593, and psilocybin were not effective at blocking the pain-depressed behavior. Subsequent studies determined the role of clinically available mu-ligands to (1) cause motor disruption when tested alone, and (2) alleviate the IP acid-induced depression of behavior. Results from these studies suggested that mu-ligand

effects to cause motor disruption when tested alone were dose- and efficacy dependent, and the greatest window to determine alleviation of the pain-depressed behavior was with intermediate-efficacy opioids. These results provided the pipeline for the studies conducted in **Chapter 5** to test the novel single-molecule opioids in the locomotor + barrier assay.

In **Chapter 5**, two initial studies were performed before we initiated testing with a series of the novel single-molecule opioids in the locomotor + barrier assay. First, two different investigators (1 male and 1 female) tested a range of IP lactic acid concentrations (0.18-0.56%), and results showed no effect of investigator. This is important because studies suggest that male investigators may affect rodent behavior more than female investigators; however, we did not see that in this study (Sadler et al., 2021; Sorge et al., 2014). This absence of an investigator sex effect may reflect the short amount of time that investigators were in the room to inject mice and place them in the chambers for testing and isolation of mice in sound-attenuating chambers during testing. Because there was good inter-investigator reliability in experimental outcomes, subsequent studies with test drugs were conducted by one or the other investigator, and the highest concentration tested (0.56%) of IP acid was used because it produced the most robust behavioral effects.

Second, the clinically available intermediate-efficacy opioid buprenorphine was tested (1) alone, and (2) in the presence of IP acid on the two behavioral endpoints: (1) “Crosses” defined as the number of crosses between the compartments, which requires mice to rear and surmount the vertical barrier in the doorway, and (2) “Movement” defined as the total number of beam breaks in each individual compartment, which requires only

horizontal locomotor activity. Results indicated that buprenorphine alone was effective to increase crosses but not movement, and that buprenorphine was able to alleviate the IP acid-induced depression of crosses and movement. These results on horizontal and vertical activity contrast with effects reported in **Chapters 2** and **4** with buprenorphine and illustrate the importance of experimental context for expression of opioid effects on activity. Thus, in **Chapter 2**, horizontal locomotor activity was evaluated in a rectangular open field during 60-min sessions. This session duration provided an opportunity for high initial activity to decline as mice habituated to the chamber, and buprenorphine produced a hyperlocomotor effect not by increasing initial high rates of activity, but rather by delaying habituation. By contrast, in the present study, locomotor activity was assessed in a more complex 2-compartment chamber during shorter 15-min sessions, and under these conditions, buprenorphine did not increase the measure of horizontal activity (i.e. "Movement"). Similarly, crosses in the locomotor + barrier assay requires a modest expression of vertical activity to surmount the barrier, but buprenorphine effects on crosses in this study contrasts with its effects on climbing in the vertical chamber used in **Chapter 4.** In the present study, buprenorphine increased the number of crosses at the highest doses tested (0.1-0.32 mg/kg); however, in the climbing study (**Chapter 4**), buprenorphine decreased climbing at the highest dose tested (0.32 mg/kg). Taken together, these results with buprenorphine indicated that the locomotor + barrier procedure was more resistant to opioid-induced disruption and more sensitive to opioid analgesia than the locomotor or climbing procedures described in the earlier chapters. Accordingly, we proceeded to evaluate the effects of the novel single-molecule MOR

agonists with graded MOR efficacies described in **Chapter 3** (Chambers et al., 2022; Gutman et al., 2020, Lutz 2023; *In Press*, Tom Prinsenzano personal communication).

Results indicated that when the opioids were tested alone, their ability to alter crosses and movement was dependent on their efficacy. Only the highest efficacy opioid DC-01-128.1 decreased behavior, whereas several of the intermediate-efficacy opioids (DC-01-76.2, EWB-3-14, JL-2-39) modestly but significantly increased either crosses or movement, and the lower efficacy opioids (DC-01-76.1, EG-1-203, EG-1-230) had no effect on either crosses or movement. These results provide additional evidence to suggest that activity in this procedure was less sensitive than either single-chamber locomotion or climbing to opioid-induced disruption, and this provided an opportunity to evaluate opioid effects on IP acid-induced behavioral depression. When the opioids were tested in the presence of IP acid, alleviation of the pain-depressed behavior was also efficacy dependent, with optimal effects for the intermediate-efficacy opioids. Thus, the highest two efficacy novel opioids (DC-01-128.1, DC-01-76.2) significantly alleviated IP acid effects only on movement but not on crosses, suggesting that motor disruption by these two compounds may have interfered with their effectiveness to restore crossing behavior. At the other extreme, the two lowest efficacy novel compounds (EG-1-203, EG-1-230) failed to alleviate IP acid-induced depression of either crosses or movement, suggesting that the efficacy of these compounds was too low to produce significant antinociception. Between these extremes, the remaining compounds (EWB-3-14, JL-2-39, DC-01-76.1) all produced an antinociceptive alleviation of IP acid-induced depression of both crosses and movement. Of particular note, DC-01-76.1 attenuated IP acid effects on both crosses and movement while having no effect on either endpoint when

administered alone. Thus, optimal antinociceptive restoration of pain-depressed behavior without signs of behavioral disruption was produced by the intermediate-efficacy MOR agonists and especially by DC-01-76.1.

Taken together, the results of **Chapters 4** and **5** suggest that MOR agonist effectiveness to alleviate pain-related behavioral depression depends in part on sensitivity of the target behavior to disruption by the MOR agonist administered alone. Consistent with this interpretation, IP acid in mice produces a pain-related depression of both horizontal locomotor activity (Stevenson et al., 2009) and vertical climbing behavior (Chapter 4); however, MOR agonists are less effective to decrease horizontal activity than climbing and correspondingly more effective to alleviate IP acid-induced depression of horizontal activity than climbing (Stevenson et al., 2009). As another example, IP acid also produces a pain-related depression of positively reinforced operant behavior maintained in rats by delivery of either food or a social reinforcer (brief access to another rat); however, MOR agonists are less effective to disrupt responding maintained by food than by the social reinforcer and correspondingly more effective to alleviate IP acid-induced depression of food- than social-maintained responding (Baldwin et al., 2022). This interpretation has implications not only for MOR agonist effects in preclinical assays of pain-depressed behavior, but also for clinical effects of MOR agonists in humans pain patients. Pain states can interfere with a variety of behaviors in humans, and opioid analgesic effectiveness to alleviate pain-related behavioral depression may also be influenced by the behavioral endpoint of interest and the sensitivity of that endpoint to disruption by the opioid.

7.6. Part III – Expression of chronic pain.

Chapter 6 focused on studying the effects of three different experimental chronic pain manipulations in the locomotor + barrier assay. The experimental chronic pain models studied here differ along multiple dimensions including (1) the mechanism by which they activate nociceptive signaling (**Introduction; section 1.8**), (2) the duration of behavioral effects they produce, and (3) the peak magnitude of behavioral effects they produce.

The effects of CFA reported here agree with previous reports of increased and sustained paw-width inflammation in mice (Cobos et al., 2012). For example, they found that 20 µl of CFA was sufficient to increase paw-width for up to seven days compared to the saline control group. Although CFA produced sustained paw-width inflammation at all three time points studied here (6-, 24-, and 72-hours) post-injection, CFA did not decrease the number of crosses but produced a weak decrease in overall movement. This decrease in movement also agrees with previous studies with its effectiveness to decrease other types of locomotion such as voluntary wheel running (Cobos et al., 2012), locomotor activity (Sheahan et al., 2017), and nesting (Negus et al., 2015).

Laparotomy was evaluated using an extended 60-min behavioral session, and data analysis examined effects during the first 15 min (to be comparable to all other experiments using this procedure) and for the whole 60 min (to determine if longer behavioral sessions might alter sensitivity to laparotomy effects). When the first 15 min of data were evaluated, only movement counts were decreased but not the number of crosses. However, when data for the entire 60-min session were analyzed, there was a significant decrease in both the number of crosses and overall movement. A previous

study has shown that laparotomy was effective to decrease certain behaviors in mice such as wheel running over a period of two-hours, burrowing over a period of 30-minutes, and home cage-lid hanging over a period of 30-minutes (Ulker et al., 2022). This suggests that longer behavioral sessions in our current behavioral procedure may be necessary (as seen with results at the 60-minute timepoint) to study other chronic pain models in order to observe more robust, sustained, and significant effects. This idea is supported by other reports suggesting that mice may need up to 24-hours of uninterrupted behavioral time in order to see a decrease in locomotor behavior (Sheahan et al., 2017; van't Land and Hendriksen, 1995). Another implication of this extended time is the idea that short sessions (like the 15-minute behavioral sessions used in most of our studies) may not be long enough to allow mice to go through the onset exploratory behavior of a new environment. In some cases (e.g. wheel running), acclimation to the behavior and/or environment may be necessary, but in the current study, mice are not acclimated to the locomotor + barrier boxes so that baseline behavioral rates are sufficiently high and insensitive to pain-related depression. Longer behavioral sessions may allow us to both retain the high rates of behavior observed during initial exposure to the chambers while also sampling behavior at later times to assess the impact of pain manipulations on lower but still significant behavioral rates as mice acclimate to the chamber.

The effects of SNI reported here agree with previous reports of increased and sustained mechanical hypersensitivity in mice over a period of weeks (Inyang et al., 2019). For example, the Inyang et al. 2019 study is one of the few that includes both male and female outbred strain of mice, and it shows sustained mechanical hypersensitivity for up to 56 days post-surgery in both sexes. Although SNI produced sustained mechanical

hypersensitivity in the current study, SNI only had an effect on movement but not crosses; however, the effect on movement was sustained for the entire period of testing. This slightly contrasts with previous findings, which did not find a significant SNI effect on open-field locomotor behavior or voluntary wheel running but did find sustained mechanical hypersensitivity for up to 40 days post-surgery (Sheahan et al., 2017). However, it is important to note two methodological differences between these studies. First, the findings in Sheehan et al. 2017 were based on testing male C57BL/6J mice, thus presenting a sex and strain difference. Second, the Sheehan et al. 2017 study used a rectangular open field environment, whereas the present study used a more complex locomotor setting that involves a combination of horizontal and vertical movement. Another important contribution of our current study is the time to test post-surgery. According to a previous survey of the literature (Millecamps et al., 2023), there are 23 published papers (2000-2022) in which SNI animals have been tested for more than 3 months (12 weeks) post-surgery. The present study adds to this list of 23 publications because our SNI animals underwent behavioral testing for 105 non-consecutive days (15 weeks) at an average age of 7 months. This is an important point to consider because the average post-injury time at which SNI animals get tested is about four weeks (Zhang et al., 2020) , and we know that chronic pain manifestation is considered as pain persisting for more than ≥3 months in humans. Now the implication of what a “mouse lifespan” is compared to a “human lifespan” is an ongoing debate, but there is a growing body of evidence to suggest that longer test time points should be considered in these types of chronic pain manipulations (Millecamps et al., 2023; Muralidharan et al., 2020).

A final implication of the relatively weak effects reported here is the idea that adult human nociceptors may be different than rodent nociceptors (Walters et al., 2023). For example, human nociceptors appear to have a peptidergic phenotype (i.e. expressing peptides like calcitonin gene-related peptide as well as TRPV1), whereas rodent somatic nociceptors include both peptidergic and nonpeptidergic (i.e. expressing the lectin IB4) subclasses that appear to mediate the behavioral effects of thermal vs. mechanical noxious stimuli, respectively. Notably, rodent deep-tissue and visceral nociceptors appear to be primarily peptidergic, suggesting that visceral pain models in mice may activate peptidergic nociceptors most homologous to human nociceptors, whereas somatic pain models, and especially somatic pain models relying on mechanical stimuli, may activate nociceptors that are not homologous to human nociceptors. An additional species difference in nociceptors is that human nociceptors express multiple types of acid-sensing ion channels (ASICs), whereas rodent nociceptors express primarily the ASIC3 subtype. These species differences in nociceptor phenotype have been interpreted to suggest that humans may have a higher propensity than rodents for nociceptor hyperactivity leading to greater functional impairment as reported in human chronic pain cases (Walters et al., 2023).

7.7. The role of sex as a biological variable

None of the studies in this project were intended *a priori* to detect sex differences in either basal behavioral effects or treatment effects on behavior; however, in accordance with National Institutes of Health Guidelines (Miller et al., 2017), the studies presented did include both male and female subjects and included both inferential statistical analysis and post-hoc power analysis as we have described previously to

assess the role of sex as a biological variable (Diester et al., 2019). Throughout the work presented in this dissertation, in general, when sex effects were determined they were weak, and they will be discussed below.

Sex Differences in MOR Agonist-Induced Hyperactivity. In locomotor activity studies reported in **Chapters 2** and **3**, evidence for sex differences was weak and did not vary systematically as a function of MOR efficacy. Studies with most drugs and mixtures found only small effect sizes for the main effect of sex or the sex × dose interaction, and post hoc power analysis indicated that most group sizes were underpowered to detect significance of any sex differences that might actually exist. In the cases where the main effect of sex or sex × dose interaction was significant, locomotion tended to be higher in males, but this could not be attributed to higher sensitivity to opioid-induced hyperactivity because the sex × dose interaction either was not significant or was not followed by a significant post hoc effect of sex at any dose. Previous studies have also found little evidence for sex differences in opioid-induced hyperactivity in mice. Main effects of sex have been observed suggestive of different baseline levels of activity, but the sex showing higher activity has varied (Collins et al., 2016; Kavaliers and Innes, 1987; Szumlinski et al., 2020). We could find only one study to show a significant sex × dose interaction with a significant post hoc sex difference, with male deer mice showing higher activity than females during the light phase after treatment with 1 mg/kg of morphine (Kavaliers and Innes, 1987).

Sex as a determinant of treatment effects on pain-depressed behavior. In **Chapter 4** studies, in most test groups, there was not a main effect of sex or a significant sex x dose interaction, which implies little or no role of sex as determinant of climbing. In

the three groups that did display a main effect of sex, the males climbed less than the females. In **Chapter 5** studies, in general, sex effects that were determined were not efficacy dependent or dependent on whether the behavior was studied after administration of drug alone or in the presence of the pain stimulus. In the five groups that did display a main effect of sex, the males showed more crosses and movement than the females. Thus, in the two assays of pain-depressed behavior studied (climbing; **Chapter 4** and locomotor + barrier; **Chapter 5**) the direction of the behavior expressed went in different directions within the same sex. An implication of this may be the experimental context for the expression of opioid effects on behavior (vertical chamber; **Chapter 4**) and (horizontal chamber + small vertical barrier; **Chapter 5**).

Sex effects of chronic pain-depressed behavior. The only significant sex effect identified in **Chapter 6** was in the inflammation associated with increased paw-width in the CFA treated animals. A sex effect was not detected in the CFA, laparotomy, or SNI groups in either the number of crosses or overall movement. Interestingly, (Millecamps et al., 2023) found a sex effect in mechanical hypersensitivity in mice after SNI surgery at 6- and 9-months post-surgery, with male mice showing greater mechanical hypersensitivity. However, that study was done in C57BL/6J mice that were purchased from a vendor or bred in-house. In comparison, our studies were done in ICR mice purchased from a vendor.

Throughout the work presented in this dissertation, when sex differences in effects of a manipulation were observed, the effects of the manipulation were greater in male mice. Moreover, the relatively weak evidence for sex differences was evident with opioid effects alone, for the pain stimulus alone, and for opioids in the presence of the pain

stimulus. This agrees with conclusions made by (Dahan et al., 2008), which determined after a systematic review of the literature that (1) there is no clear consensus to determine which sex is more sensitive to opioid effects and opioid analgesia, (2) determination of sex differences needs consideration of a variety of independent variables such as: strain, age, opioid [efficacy, dose, route of administration] pain stimulus, and behavior, to name a few. Results from the human literature (as described in the **Introduction; section 1.9**) suggest that women may be more sensitive to opioid effects; however, (Dahan et al., 2008) make the same point regarding the variety of independent variables that should be considered in clinical trials such as age, opioid/addiction history, opioid dose, the main dependent measure of interest, route of administration, and patient-controlled analgesia (PCA) studies. Our studies were not powered *a priori* to detect sex differences; however, as described in the **Introduction; section 1.9**, our experimental design and data analysis strategy allowed us to conduct *post hoc* power analyses that could be used to investigate sex differences in more detail. Thus, conclusions made should be considered tentative because post hoc power analysis throughout our studies indicated that power was often less than the criterion level of 0.8 commonly required to protect against a Type II error (i.e. concluding the absence of a sex difference when one is present). Two final points (1) the power analyses described here can be used to guide future studies that do choose to investigate sex differences, and (2) although the sex effects that were determined throughout the work presented in this dissertation were weak, sex as a biological variable should still be an important variable for investigators to consider.

7.8. Conclusions and future directions

The work presented in this dissertation aimed at studying MOR efficacy effectiveness in treatment of pain-depressed behavior. We focused on MOR efficacy for two main reasons. First, the current ongoing opioid epidemic has hindered the continued further research of opioids as analgesics, and while I acknowledge the crisis, I would like to also acknowledge that the main drivers of this crisis have been high-efficacy opioids such as fentanyl and oxycodone. Second, there are a few clinically available intermediate/low efficacy opioids (i.e. buprenorphine and nalbuphine) that are useful analgesics. Thus, this provides proof-of-concept that intermediate/low efficacy opioids may be better candidate analgesics. However, as noted in the work presented in this dissertation, the lack of MOR selectivity makes these compounds less than favorable. Therefore, we were interested in studying a series of intermediate- to low- efficacy opioids with our working hypothesis being that low-efficacy opioids would provide the greatest window of opportunity to determine antinociception at doses that do not cause motor impairing effects in mice (as one opioid side effect). Overall, conclusions can be broken down to two main parts. First, a series of MOR-selective intermediate- and low-efficacy opioids (with E_{max} values below buprenorphine but higher than naltrexone) produced alleviation of the pain-depressed behavior. We studied eight novel single-molecule opioids, and out of these, three (EWB-3-14, JL-02-0039, and DC-01-76.1) show the most promise, as described below. Second, we hypothesize that pain-depressed behaviors serve as better preclinical-to-clinical translational models and should be highly considered when studying novel analgesics. Again, our results support this hypothesis because we were able to determine alleviation of the pain-depressed behavior; however, as discusses

in the **Introduction; section 1.6**, some opioids may or may not be effective to block pain-depressed behaviors. Reasons why this may be includes animal strain, type of pain stimulus, intensity of the pain stimulus, or the behavior that is being measured. Our results suggest that opioid efficacy can be added to this list of variables that plays a role in the effectiveness of opioids to block pain-depressed behavior.

As the author of this dissertation, I believe that this project could be lead in a few different directions. First, because a subset of novel MOR-selective opioids from **Chapter 5** supported the working hypothesis, they should be studied in our chronic experimental pain models. To be more specific, out of the eight novel opioids studied, in my opinion, three show the most potential. Those three are EWB-3-14, JL-02-0039, and DC-01-76.1 for two reasons. First, they have lower E_{max} values than buprenorphine (but higher than naltrexone), and second, these three compounds showed two effects (1) less motor disruption when tested alone, and (2) a greater potency difference to produce antinociception vs motor disruption (if any). Second, because Chapter 6 showed depression of movement, specifically, in all treatment groups (CFA, laparotomy, SNI), I would study the three novel opioids described above to determine opioid effectiveness to reverse these examples of experimental chronic pain-depressed behavior.

The work presented in this dissertation focused on a variety of locomotor behaviors as one opioid side effect in mice. However, we know that opioids produce a myriad of side effects such as GI inhibition, lethal respiratory depression, pruritus, and abuse liability; however, I would like to note that to be able to study this was out of the scope of my dissertation. Thus, as an important future direction should be to test all the novel single-molecule opioids to determine their effects on other opioid related side effects.

While all novel opioids are of interest, I would again start with the three opioids chosen above (EWB-3-14, JL-02-0039, and DC-01-76.1). Specifically, it would be of interest to determine if these compounds are able to reach the ceiling respiratory depressant effects observed with buprenorphine, and if tolerance builds to the GI inhibitory effects. Ultimately, the goal would be to move (at least) one of these compounds into clinical human trial studies to determine (1) adverse effects, (2) pharmacokinetics, and (3) analgesia in patients suffering from pain.

After I defend my dissertation, I will be moving on to a postdoctoral position at Duke University School of Medicine at the Center for Translational Pain Medicine in the Department of Anesthesiology. I will be joining the Human Affect and Pain Neuroscience (HAPN) Lab which is directed by Dr. Katie Martucci. The HAPN Lab uses magnetic resonance imaging (MRI) as a tool to study the brain and spinal cord to identify changes in structure and activity in patients suffering with chronic pain. A main project that I will be working on as a postdoc will be on identifying how opioids affect the neurophysiology of the central nervous system (CNS) in patients taking opioids to treat their chronic pain. As stated earlier in this section, in my opinion, three out of the seven novel opioids show the most promise; therefore, hypothetically, if I had to choose one opioid to study during my postdoc it would DC-01-76.1 because it is (1) mu-selective, (2) a low-efficacy opioid (lower than buprenorphine), (3) did not produce motor disruption at doses tested, and (4) produced antinociception in both behavioral endpoints (crosses and movement) in an assay of pain-depressed behavior in preclinical studies. Thus, it would be interesting to determine how DC-01-76.1 affects and/or alters the CNS neurophysiology of pain, and if

it can restore the pain related behavioral depression that is associated with high-impact chronic pain.

My experience with pain

As many of you know, I have suffered with chronic pain for as long as I can remember. My official last diagnosis was low-back pain with sciatica related symptoms. Unfortunately, my experience with "pain" physicians has not been great. The last time I saw a pain doctor was in 2021 and I have not gone back since. The current first-line diagnostic tools for pain patients include the numerical rating scale and the visual analog scale, and as a patient, I can share that I am not a fan of these rating scales. They do not encompass the pain experience and they are tools that provide a fast answer for a long and complicated issue. Because of the severity of my pain, I was prescribed gabapentin (three different times, when I explicitly said no to the previous time). My reasoning for not taking the gabapentin is beyond this summary, but I am sharing this to hopefully provide a glimpse as to how pain patients can be treated. So, how is my pain now? Well, since I didn't take the gabapentin, the next "treatment" I was offered was physical therapy. And while that seemed to help, it was not enough. Nowadays, I am managing my pain by strength training, heating pads, and Icy Hot. Lifting heavy seems to calm down my nerve and my flares; however, when I do have flares, they are bad enough that sometimes I just want to cut my leg off (sorry for the explicit image). But the pain can be severe enough that I have functional impairment, and for me that usually means I stop exercising (which I absolutely love to box, walk, run, lift weights), I lose my appetite, my sleep is restless, and my work is affected. To share my last thought with you: my ideal pain treatment would be the following, because my pain is "localized" [either low-back, hip, or right leg] I would love a medication that can target where I feel pain *at that* moment without producing side effects, specifically motor impairment, and 2) continued strength training that reduces the

flare-ups and targets areas where the sciatic nerve runs through. I am sharing my experience because the work I dedicated the last 5 years of my life was not just “work” so that I could obtain a degree, but it was work and hypotheses that I as a scientist and pain patient (I always call myself the N=1) believed could further pain medicine and treat millions of patients (like me).

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Vita

Edna Santos was born in Guatemala City, Guatemala on September 10, 1992, but was raised in Los Angeles, California from the age of 7 months. She graduated from high school in 2011. She then went on to complete her Associate of Arts degree in Natural Sciences and Mathematics in 2016 from Los Angeles City College. She then transferred to Virginia Commonwealth University in 2016 and completed her Bachelor of Science degree in Biology in 2018. She went on to pursue graduate studies at Virginia Commonwealth University in 2018 to obtain her PhD in Pharmacology under the guidance of the best mentor, Dr. Steve Negus.

Edna J. Santos

EDUCATION

2018-2023 Virginia Commonwealth University
PhD in Pharmacology and Toxicology

2016-2018 Virginia Commonwealth University
B.S. in Biology

2011-2016 Los Angeles City College
A.A. in Natural Sciences and Mathematics

EXPERIENCE

Department of Pharmacology and Toxicology - VCU

Graduate Research Student 2019 - 2023
Mentor: Dr. S. Stevens Negus
Proposed thesis work: "Intrinsic Efficacy as a Determinant of Opioid Effectiveness in Treatment of Pain-Depressed Behavior."

Graduate Research Student 02/19 – 05/19
Mentor: Dr. S. Stevens Negus
"Application of Receptor Theory to the Design and Use of Cannabinoid Receptor Agonist and Antagonist Mixtures in Mice."

Graduate Research Rotating Student 11/18 - 02/19
Mentor: Dr. Hamid Akbarali
"The Effect of Loperamide, a Peripheral Opioid Agonist, on Morphine Tolerance."

Graduate Research Rotating Student 08/18 - 11/18
Mentor: Dr. Aron Lichtman
"CP55940 Dose Dependently Produces Antinociception in an Acute Pain Mouse Model."
Summer Research Student 07/18 - 08/18
Mentor: Dr. Hamid Akbarali
Observed mouse DRG extraction and learned electrophysiology basics.

Department of Neurology - VCU

Undergraduate Research Assistant 05/17 - 05/18
Mentor: Dr. Laxmikant Deshpande
Investigated the effects of ketamine and levetiracetam in an organophosphate rat model of Gulf War Illness.

POSTER RESENTATIONS

2023 The United States Association for the Study of Pain (USASP)
Durham, NC
Edna J. Santos*, Nima Nassehi*, Eric W. Bow, Dana R. Chambers, Eugene S. Gutman, Arthur E. Jacobson, Joshua A. Lutz, Kenner C. Rice[2] Agnieszka Sulima, Dana E. Selley, Young K. Lee, and S. Stevens Negus. "Efficacy as a Determinant of Mu Opioid Receptor Analgesic Effects in a Novel Assay of Pain-Depressed Behavior in Mice. II. Effects of Novel Low-Efficacy MOR Agonists"
*Co-First authors

2022 The United States Association for the Study of Pain (USASP)
Cincinnati, OH
Edna J. Santos, Arianna Giddings, Farah Kandil, S. Stevens Negus.
"Expression and Treatment of Pain-Depressed Climbing in Male and Female Mice"

2022 Women's Health Research Day
VCU – Richmond, VA
Edna J. Santos, Arianna Giddings, Farah Kandil, S. Stevens Negus.
"Pain-depressed climbing in Male and Female Mice as a Tool for Analgesic Drug Development"

2021 The International Narcotics Research Conference (INRC)
Virtual
Edna J. Santos, Matthew L. Banks, S. Stevens Negus. "Role of Efficacy as a Determinant of Locomotor Activation Induced by Mu Opioid Receptor Ligands in Male and Female Mice"

2021 ASPET Experimental Biology (EB)
Virtual
Edna J. Santos, Matthew L. Banks, S. Stevens Negus. "Role of Efficacy as a Determinant of Locomotor Activation Induced by Mu Opioid Receptor Ligands in Male and Female Mice"

2018 VCU Poster Symposium for Undergraduate Research and Creativity
VCU - Richmond, VA
Santos, E. Church, E. Deshpande, L. Gray, M. Hawkins, E. Phillips, K. Vu, E.
"Levetiracetam ameliorates depression and cognitive deficits in a DFP-based rat model of Gulf War Illness."

2018 Central Virginia Chapter of the Society for Neuroscience (CVCSN)
VCU - Richmond, VA
Santos, E. Church, E. Deshpande, L. Gray, M. Hawkins, E. Phillips, K. Vu, E.

"Levetiracetam ameliorates depression and cognitive deficits in a DFP-based rat model of Gulf War Illness."

2018 Society of Toxicology (SoT)
San Antonio, TX
Santos, E. Church, E. Deshpande, L. Gray, M. Hawkins, E. Phillips, K. Vu, E.
"Levetiracetam ameliorates depression and cognitive deficits in a DFP-based rat model of Gulf War Illness."

TALKS

2022 Center of Health Disparities (CoHD) Research Retreat
Richmond, VA
Invited speaker as IMSD PhD Alumni: "Expression and Treatment of Pain-Depressed Climbing in Male and Female Mice"

2022 The European Pain School (EPS)
Siena, Italy
"Expression and Treatment of Pain-Depressed Climbing in Male and Female Mice"

20-23 Pharmacology Departmental Student Seminar
VCU - Richmond, VA
"Intrinsic Efficacy as a Determinant of Opioid Effectiveness in Treatment of Pain-Depressed Behavior."

2019 Near Peers Seminar Series
VCU - Richmond, VA
Invited talk: "From LA to RVA: Responding to the Opioid Crisis"

PUBLICATIONS

1. **Santos EJ**, Giddings AN, Kandil FA, Negus SS. Climbing Behavior by Mice as an Endpoint for Preclinical Assessment of Drug Effects in the Absence and Presence of Pain. Frontiers in Pain Research, 2023.
2. **Santos EJ**, Banks ML, Negus SS. Role of efficacy as a determinant of locomotor activation by mu opioid receptor ligands in female and male mice. J Pharmacol Exp Ther, 2022. PMID: 35489781
3. Baird TR, Akbarali HI, Dewey WL, Elder H, Kang M, Marsh SA, Peace MR, Poklis JL, **Santos EJ**, Negus SS. Opioid-Like Adverse Effects of Tianeptine in Male Rats and Mice. Psychopharmacology, 2022. PMID: 35211768
4. Ma H, Li M, Pagare PP, Wang H, Nahessi N, **Santos EJ**, Negus SS, Selley DE, Zhang Y. Novel Bivalent Ligands Carrying Potential Antinociceptive Effects by Targeting Putative Mu Opioid Receptor and Chemokine Receptor CXCR4 Heterodimers. Bioorganic Chemistry, 2022; 120 (105641) PMID: 35093692
5. Diester CM, **Santos EJ**, Moerke MJ, Negus SS. Behavioral Battery for Testing Candidate Analgesics in Mice. I. Validation with Positive and Negative Controls. J Pharmacol Exp Ther, 2021; 377 (2) 232-241 PMID: 33622770
6. Selley DE, Banks ML, Diester CM, Jali AM, Legakis LP, **Santos EJ**, Negus SS. Manipulating Pharmacodynamic Efficacy with Agonist + Antagonist Mixtures: In Vitro and In

Vivo Studies with Opioids and Cannabinoids. J Pharmacol Exp Ther, 2021; 376 (3) 374-384 PMID: 33443077

7. Phillips K, **Santos E,** Blair RE, Deshpande LS. Targeting Intracellular Calcium Stores Alleviates Neurological Morbidities in a DFP-Based Rat Model of Gulf War Illness. Toxicol Sc, 2019; 169:567- 678, PMCID: 6542335

LABORATORY SKILLS

- Behavioral assessments in mice
  - Pain-related behaviors
    - Pain-stimulated behaviors: acid-induced stretching, facial grimace, hot-plate paw-withdrawal, and warm water tail-withdrawal
    - Pain-depressed behaviors: acid-induced depression of climbing, nesting, and rearing
  - Locomotor behavior
  - Complete Freund's Adjuvant (CFA) hind paw administration
  - Spared nerve injury (SNI) surgical model of neuropathic pain
  - Laparotomy model of post-surgical pain
- Behavioral assessments in rats
  - Depression-related behaviors
    - Forced-swim test
  - Anxiety-related behaviors
    - Elevated plus maze
- Drug preparation and injection by multiple routes of administration in mice
- In vitro assessment of receptor function with assay of agonist-stimulated [^{35}S]GTPγS binding
- Data analysis and graphing using Microsoft Excel and Prism

GRANTS, HONORS AND AWARDS

2022-23 F31 NINDS Predoctoral Individual Fellowship from the National Institutes of Health (NIH)
2023 USASP Travel Award
2022 July Highlighted Trainee Author of the *Journal of Pharmacology and Experimental Therapeutics*
2022 VCU Dept. of Pharmacology and Toxicology Anthony Ambrose Award
2022 VCU Phi Kappa Phi Susan E. Kennedy Graduate Award
2022 VCU School of Medicine (SoM) Susan E. Kennedy Supplemental Scholarship
2022 VCU School of Medicine Travel Award
2022 European Pain School (EPS)
2021 ASPET Experimental Biology (EB) Travel Award
2018-20 Initiative to Maximize Student Development (IMSD) PhD Fellowship (NIH)
2018 Los Angeles City College (LACC) Invited Guest Travel Award
2018 VCU Biology Travel Award
2017 VCU Omicron Delta Kappa Honors Society
2017 VCU Tau Sigma National Honors Society
2013-16 LACC Ralph Bunche Scholars Program
2013 LACC Phi Theta Kappa International Honors Society

ADVISORY BOARD

2021-2023	VCU Women in Science President (2022-23); Secretary/VP of Communications (2021-22)
2022	VCU SoM Teaching Excellence Awards Selection Committee Graduate Student Representative
2022-Present	United States Association for the Study of Pain (USASP) Education and Professional Development Committee
2020	VCU Dept. of Pharmacology and Toxicology Diversity Committee Member
2019-20	VCU Pharmacology and Toxicology Student Organization Secretary
2018-19	VCU Omicron Delta Kappa Leadership Organization Alumni Liaison

TEACHING / MENTORING

2023	Teacher's Assistant (TA) in PHTX697: Directed Research in Pharmacology
2020-22	Mentored two undergraduate pre-medical students at VCU

PROFESSIONAL AFFILIATIONS

2021	United States Association for the Study of Pain (USASP)
2021	International Narcotics Research Conference (INRC)
2021-22	AAAS/Science Program for Excellence in Science
2020	American Society for Pharmacology and Experimental Therapeutics (ASPET)
2020	International Association for the Study of Pain (IASP)
2019-20	Journal of Opioid Management
2018-19	Society of Toxicology (SOT)

OTHER

- JPET Highlighted Trainee of the Month information: http://ow.ly/geMU50JW87Q
- Languages – Spanish (written, spoken)