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Pharmacological assessment of adjuncts to enhance mu-opioid receptor agonist antinociception in male rhesus monkeys: Does one + one = three?

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Abstract

Mu-opioid receptor (MOR) agonists are effective agents for pain management, but are also limited by a number of undesirable effects. One approach to enhance the therapeutic effects and minimize the undesirable effects of MOR agonists may be to combine MOR agonists with an adjunct targeting a different receptor system. This targeted medical approach, known as "combination therapy", aims to augment the desired effects of the MOR agonist (i.e. antinociception) and/or diminish the undesirable deleterious side effects of the MOR agonist. This dissertation investigated the utility of this approach in an assay of thermal nociception and schedule-controlled responding in male rhesus monkeys with three aims. One aim determined the utility of N-methyl D-aspartate (NMDA) receptor antagonists to selectively enhance MOR agonist antinociception. A second identified the pharmacological determinants of antinociceptive interactions between a nociceptin opioid peptide (NOP) receptor agonist and MOR agonists. A third aim investigated the potential for fixed-proportion mixtures of a competitive MOR antagonist and MOR agonist to manipulate antinociceptive efficacy. Experimental results did not support the utility of NMDA antagonists as adjuncts to selectively enhance MOR agonist antinociception. Furthermore, the antinociceptive interactions between a NOP agonist and MOR agonists were modest and occurred under a narrow range of conditions. Finally, fixed proportion MOR antagonist-agonist mixtures were effective in manipulating antinociceptive in vivo efficacy. In conclusion, this dissertation does not provide strong empirical evidence that a combination therapy approach will result in clinically effective and selective enhancement of MOR agonist analgesia. The dissertation concludes with proposed strategies and novel preclinical methods to enhance preclinical-to-clinical translation of effective candidate analgesics.

Chapter 1: Introduction

Pain as a Problem

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (IASP, 2017). Pain, and the management thereof, remains a significant healthcare challenge in the United States and worldwide. The estimated financial burden on society associated with patients suffering from some aspect of pain is over 550 billion dollars annually (Gaskin and Richard, 2012). Moreover, nearly one-third of Americans reported at least one pain symptom within the past three months, and nearly one-fifth of American adults are afflicted with chronic pain (Nahin et al, 2015; Dahlhamer et al, 2018). Chronic pain is defined as pain persisting for greater than one month, and it is often comorbid with various other disorders, ranging from arthritis to cancer (IASP, 2017). Additionally, pain symptoms are a common reason to visit a medical professional in developed countries (Schappert et al, 2006). Furthermore, between 2006 and 2016, more than 215 million opioid-based prescriptions were written each year (CDC, 2018). Unfortunately, this high rate of opioid prescriptions has contributed to the current opioid crisis in the United States (Delgado et al, 2018). Taken together, the financial, societal, and individual burdens emanating from pain and its treatment highlight the need for preclinical research to develop novel analgesics and/or strategies to enhance the therapeutic effects and minimize the undesirable effects of opioids for pain management.

Brief History on Opioid Agonists as Analgesics

Opioid agonists are one of the longest used psychoactive substances known to man, save for alcohol and marijuana. However, throughout much of human history, opioid use was limited to the opium poppy (*Papaver somniferum*), and until relatively recently, the plethora of opioid

compounds currently available to modern medicine remained undiscovered. While some archaeological discoveries of *Papaver* date back over 8000 years, the earliest evidence of cultivation and use can be traced back to Sumer in 3400 BCE based on the discovery of stone tablets referring to the opium poppy as "Huy Gil", which is roughly translated to "Joy Plant" (Brownstein, 1993). This example is merely the earliest widely accepted finding, as nearly every major society after Sumer has some evidence of opium utilization either for recreational or medicinal purposes (Brownstein, 1993; Presley and Lindsley, 2018).

While nearly a millennium would pass before a scientific understanding of the nature by which opium produces its abuse-related and medicinal effects would arise, opium was readily utilized during antiquity. Knowledge of the medicinal properties of the opium poppy quickly disseminated from Sumer to the Assyrians to the Egyptians to the Grecians, etc. During these times, opium use was most commonly associated with ritualistic and religious rites, as evidenced by various fables of its bestowment upon man by various gods and their close association with poppies in numerous visual representations (Schiff Jr, 2002). However, there was some evidence of opium use for medicinal purposes, namely for euthanasia, when opium would be mixed into a cocktail with poison hemlock (*Conium maculatum*) (Brownstein, 1993).

The Islamic societies of the early Middle Ages were some of the first to begin rudimentary investigations into cataloging the physiological effects of opium in medical texts and initiated the introduction of opium to the Far East via the Silk Road (Brownstein, 1993; Presley and Lindsley, 2018). Moreover, the famous physician and author of *Cannon of Medicine*, Avicenna, was the first to catalog many of opium's physiological effects including: analgesia, antitussive, gastrointestinal distress, respiratory depression, neuromuscular disturbances, hypnosis, and somnolence. However, perhaps more impressive and innovative for the time, was

Avicenna's recommendations for delivery and dosages of opium. This marked some of the first instances of opium standardization for medicinal use (Heydari et al, 2013). This text and its subsequent translation to Latin was a key factor in introducing opium to Western medicine, where its medicinal potential and use blossomed during the Renaissance (Smith, 1980).

Since their initial cultivation and through the modern day, the alkaloids of interest that are present in opium poppy have been extracted in a similar manner. When the plant is ready to be harvested, the bulb is scored with a blade, and a milky wax, usually referred to as opium latex, pours from the bulb. Once the latex has dried, it is dissolved in boiling water to remove any impurities. When enough liquid has been boiled off, a waxy residue is left, called smoking opium (Presley and Lindsley, 2018). Although *Papaver somniferum* contains various alkaloids, there are six that are generally thought to contribute to the prototypical physiological and psychological effects of opioids. These six alkaloids are morphine, codeine, thebaine, narceine, noscapine, and papaverine.

Scientific Advancements to Improve Therapeutic Effects and Minimize Undesirable Effects

Following the initial isolation of morphine from opium poppy by Sertürner in 1804, there was a steady increase in the isolation of naturally occurring opioid alkaloids and synthesis of synthetic opioid compounds (Courtwright, 2009). For example, codeine was first isolated in 1832 by Robiquet. Felix Hoffman synthesized the first synthetic opioid, heroin, in 1897 (Science History Institute, 2019). Throughout the 20th century, a number of new synthetic opioid compounds were generated including: oxycodone, methadone, meperidine, fentanyl, nalbuphine, and buprenorphine (Cowan et al, 1977; Sneader, 2005). Buprenorphine's synthesis also marked one of the first attempts to intentionally improve opioid analgesics. The rationale was to create a low efficacy opioid agonist that would still produce pain relief while having an improved safety

profile (i.e. reduced incidence of respiratory depression). The drug design approach for buprenorphine lead to another experimental approach of reducing undesirable opioid side effects and increasing antinociceptive potency or efficacy by combining an opioid with an adjunct compound active at another receptor system (O'Connell et al, 2014; Li, 2019; Viscus, 2019).

The concepts of potency and efficacy are important pharmacological factors in the context of opioid pharmacology. Potency is "a measure of drug activity expressed in the terms of the amount required to produce an effect of given intensity", whereas efficacy is the maximal response produced by a drug (Neubig et al, 2003; Holford and Sheiner, 1981). For example, in the context of opioids, buprenorphine is highly potent drug as its antinociceptive effects are apparent at relatively low doses; however, buprenorphine is a low efficacy drug as it can only produce antinociception (i.e. the action of blocking the detection of a noxious stimulus) against relatively low noxious stimulus intensities (Walker, Zernig, and Woods, 1995). In contrast, methadone is not a potent drug, but is a high efficacy drug, as it will produce antinociception against relatively high noxious stimulus intensities (Mello and Mendelson, 1985; Cornelissen et al, 2019). Further, potency and efficacy are not always negatively correlated as the previous examples may suggest. For example, fentanyl is a very potent, highly efficacious opioid (Finch and DeKornfeld, 1967; France et al, 1992). Potency and efficacy are important defining factors in the overall behavioral effects of opioid drugs and have the potential to be manipulated in an attempt to improve the clinical utility of these compounds.

Drug adjuncts are secondary compounds administered in conjunction with another primary drug in a targeted medical approach known as "combination therapy". This practice has been successfully implemented for the treatment of a variety of disorders ranging from neurological to cardiovascular etiologies (Li, 2019). Moreover, adjuncts are a particularly

attractive drug development method because it allows for the potential repurposing of currently FDA-approved medications to facilitate the drug development and clinical trial processes (Li, 2019). This approach is also employed in the pain field (e.g. Vicodin), and could potentially serve as one method to enhance or retain the therapeutic effects while also minimizing the undesirable effects of opioids (Collins et al, 2018). In the context of these experiments, an adjunct, which could increase opioid antinociceptive potency, would result in less opioid agonist being administered to produce similar antinociceptive effects, whereas increasing opioid antinociceptive efficacy would be expressed as the opioid and adjunct combination producing a greater antinociceptive maximal effect compared to the opioid alone (Li, 2019).

The notion of combining an opioid with another compound(s) acting at a different receptor target is not a recent development in medicine. For example, opium was commonly combined with poison hemlock for the purposes of euthanasia in antiquity; further, *spongia somnifera*, a sponge soaked with a cocktail of: opium, mandrake, poison hemlock, and henbane (*Hyoscyamus niger)*, was utilized as an early anesthetic in Europe during the Middle Ages (Brownstein, 1993; Prioreschi, 2003). Laudanum (from the Latin meaning, "worthy of praise") is perhaps the earliest example of an opioid combination medication in direct efforts to treat pain (Potter, 1902). While laudanum's initial creation is credited to the Swiss physician Paracelsus, it is disputed as to whether or not his original "recipe" indeed included opium (Sigerist, 1941). Nevertheless, laudanum first gained notoriety as an analgesic following the English physician Thomas Sydenham's *Medical Observations Concerning the History and Cure of Acute Diseases* publication where he described the opium and ethanol tincture's exact measurements for the mixture (Davenport-Hines, 2004). Therefore, laudanum probably represents the first example of an opioid combination medication for pain management.

Regardless of whether or not the ethanol "adjunct" of laudanum augmented opium's analgesic effects, Brompton cocktail was the first opioid combination developed with that explicit goal. Brompton cocktail was comprised of: an opioid agonist (morphine or heroin), cocaine, high-percentage alcohol, chloroform, and syrup (to increase palatability) (Richardson and Baker, 1956). The Brompton cocktail was used in both post-operative and palliative care regularly during the 1920s (Clark, 2014). Moreover, the elixir was reported to be a superior analgesic compared to morphine alone in palliative care units (Melzack, Mount, & Gordon, 1979). Although Brompton cocktail did succeed in augmenting opioid agonist-induced analgesia, more recent investigations suggest this was a non-selective enhancement. For example, some heroin/cocaine dose mixtures have been shown to engender greater drug-taking behaviors than heroin alone in preclinical drug self-administration procedures (Mello et al, 1995; Negus, 2005). As attempts to identify additional opioid combination medications progressed, so did the aspiration for their ability to *selectively* enhance analgesia and even potentially mitigate undesirable opioid effects (Smith, 2008; Li, 2019).

The most typically prescribed opioid combination medications utilize oxycodone or hydrocodone as the mu-opioid receptor (MOR) agonist and a non-steroidal anti-inflammatory drug (NSAID), such as ibuprofen, or acetaminophen (e.g. Percodan(R), Percocet(R), Vicoprofen(R), Vicodin(R)) (Raffa et al, 2010; Li, 2019). Although there is limited clinical or preclinical evidence of any additive or synergistic interactions between these two classes when co-administered, one hypothesis is that NSAID-induced cyclo-oxygenase-2 (COX-2) inhibition or acetaminophen augments the opioid-mediated analgesic effect (Raffa et al, 2010). Unfortunately, NSAIDs and acetaminophen have their own undesirable effects such as gastrointestinal bleeding, cardiovascular complications, and hepatotoxicity (Sostres et al, 2010).

Thus, there is a need for preclinical research to develop and evaluate candidate opioid adjuncts that enhance the therapeutic effects and minimize the undesirable effects in the context of pain management.

There are at least four potential approaches for the opioid+adjunct to produce an "opioid-sparing effect" (which is defined as a "reduction in the opioid dose administered without a loss in analgesic efficacy" (Nielsen et al, 2017)): (1) Prolong analgesia duration, (2) enhancement of analgesic efficacy, (3) enhancement of analgesic potency, (4) minimization or elimination of undesirable opioid-related effects (Smith, 2008; Li, 2019; Viscusi et al, 2019). Currently, NSAIDs and acetaminophen are the only opioid adjuncts available in an FDA-approved prescription medication. Thus, knowledge regarding the usefulness of this approach to improve opioid analgesics is relatively limited. Accordingly, the aim of this dissertation was to examine candidate opioid agonist adjuncts to selectively modulate opioid antinociception in preclinical assays of nociception.

Basic Opioid Pharmacology and Modulation of Nociception

MOR agonists are a class of molecules that bind to and activate the MOR receptor. The MOR receptor is one of the three "classical" opioid receptors, the other two being the delta-opioid receptor (DOR) and the kappa-opioid receptor (KOR) (Stein, 2016). The different classifications of these receptors are based on their peptide structure and the preferential binding of the three main endogenous opioids to them. For example, endorphins preferentially bind to MORs, enkephalins to DORs, and dynorphins to KORs; however, these neuropeptides are not selective and there is some overlap in binding profiles across the three opioid receptors (Stein, 2016). There also exists a fourth subtype of opioid receptor, the nociceptin-opioid peptide (NOP) receptor; however, the NOP receptor is not considered a "classical" opioid receptor due to

significant differences in the peptide composition of the receptor and NOP receptor insensitivity to the opioid antagonist naltrexone (Stein, 2016; Toll et al, 2016).

All three classical opioid receptors and the NOP receptor are G-protein coupled receptors (GPCR) which, when activated, initiate a cascade of intracellular signaling events altering cellular function (Bohn et al, 1999; Stein, 2016). All opioid receptors are coupled to a specific G-protein categorized as a $G_{i/o}$ and are composed of three subunits: alpha, beta, and gamma. The alpha subunit is a GTPase, which hydrolyzes the molecule guanosine triphosphate (GTP) to release energy contained in the phosphodiester bond at the gamma phosphate of the GTP molecule. The latter two subunits form a beta-gamma complex (Goldstein et al, 1971; Manglik et al, 2012; Wu et al, 2012; Thompson et al, 2012). Ligand binding to the opioid receptor initiates an intracellular signaling cascade following a conformational change of the receptor. This allows dissociation of the alpha subunit and beta-gamma complex from the receptor. Once this occurs, the alpha subunit inhibits production of the secondary messenger cyclic adenosine monophosphate (cAMP) and the beta-gamma complex activates multiple G protein-coupled inwardly-rectifying potassium (GIRK) channels and inhibits calcium channels (Ingram and Williams, 1994; Tedford and Zamponi, 2006; Luscher and Slesinger, 2010). The beta-gamma complex also inhibits sodium channels, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, transient receptor potential vanilloid (TRPV1) channels, and acid-sensing ion channels (ASICs) in dorsal root ganglion (DRG) neurons. Furthermore, the beta-gamma complex can also modulate pre- and post-synaptic cellular excitability by inhibition of glutamate-mediated excitatory post-synaptic potentials (EPSPs) (Herz et al, 1989; Endres-Becker et al, 2007; Spahn et al, 2013; Cai et al, 2014). The net result of these intracellular events is an increase in membrane potential and decreased likelihood of an action potential (Stein, 2016).

Although mu, delta, kappa, and NOP receptors share similar signaling mechanisms, differences in receptor density and neuroanatomic locations result in differential pharmacological effects. For example, MOR agonists produce euphoria and respiratory depression, which are two of the major undesirable effects of clinically available opioid analgesics (Hamilton and Cullen, 1953; Levitt et al, 1977; Montandon and Slutsky, 2019). DOR agonists can produce convulsions, KOR agonists can produce dysphoria (Tortella et al, 1983; Wadenberg, 2003), and NOP agonists may impair learning and memory (Toll et al, 2016). Similar to the opioid receptors' overlap in function, there is also a generous overlap in the distribution of these receptors across the nervous system. As the focus of this dissertation is on modulating nociception, the most pertinent location of opioid receptors (and specifically MORs) is along the spinothalamic tract.

The anterolateral pain processing system is the most prominent nociceptive pathway responsible for transmitting noxious stimuli information from the periphery to the brain (Kandel et al, 2013). This pathway projects from primary nociceptors along primary afferents -neurons that transmit information to the central nervous system (CNS) - that synapse in the outer laminae of the dorsal horn of the spinal cord, which in turn project to the thalamus and then relays the nociceptive signal to a number of different cortical regions (Kandel, 2013). The anterolateral pain processing system includes myelinated Aδ fibers and unmyelinated C fibers, both of which carry the nociceptive signal from the periphery to the CNS. Aδ fibers and C fibers each encode different types of sensory stimulation from the periphery, and each are innervated by both high- and low- threshold mechanoreceptors. The major difference between the two is the speed at which they will conduct an action potential. Aδ fibers tend to transmit more rapidly, while C fibers more slowly, and this discrepancy is due to the differences in myelination (Aδ fibers are myelinated, and C fibers are not) (Traub and Mendell, 1988). Furthermore, the anterolateral pain

processing system is also comprised of descending efferent neurons (i.e. neurons that transmit information away from the CNS). Descending neurons project from the parabrachial nucleus and rostroventral medulla to the soma (i.e. neuronal cell body) of nociceptive afferents in the dorsal horn of the spinal cord and are responsible for modulating nociceptive signals being transmitted from the periphery (Kandel, 2013). Some opioid receptors have been shown to have similar effects on transmission of such nociceptive signals. Mu, kappa, and delta receptor activation have all been shown to inhibit nociceptive signaling along the anterolateral pain processing system at the level of the periphery (Ko et al, 1999, 2000; Butelman et al, 2004). However, the effect of NOP receptor activation is less certain. Existing data contradictorily suggest that the endogenous ligand nociceptin/orphanin FQ (N/OFQ) can both produce antinociception (Ko et al, 2002) and block MOR-, DOR-, and KOR-mediated antinociception (Chen et al, 2007). It is through modulation of nociceptive signaling by opioids and/or novel compounds along this pathway that many preclinical assays assess the utility of candidate analgesics.

Preclinical Assessment of Candidate Analgesics

The preclinical investigation of candidate analgesics or opioid adjuncts involves three major components. The first component is research subject selection. Nonhuman primates (NHPs) are the most phylogenetically similar preclinical research subjects to humans, and their utilization should enhance vertical translation from preclinical to clinical results. Furthermore, the distribution and density of opioid receptor subtypes is much more similar between NHPs and humans than rodents and humans (Weerts, 2007). For example, rodents have a higher ratio of delta:mu receptors than do humans or nonhuman primates. Beyond this, several potential MOR adjuncts have been previously shown to have differential effects in rodents than in NHPs (Weerts et al, 2007). In addition, one of the independent variables manipulated in this dissertation is

MOR agonist efficacy, and differences in MOR agonist efficacy have been reported such that NHPs are more representative of MOR agonist antinociceptive efficacy in humans than rodents (Walker et al, 1993, 1995; Maguire and France, 2014; Cornelissen et al, 2018b).

The second component is the noxious stimulus and there are numerous types of experimental noxious stimuli. This dissertation utilized a thermal noxious stimulus, specifically warmed water. In a warm-water tail withdrawal procedure, a subject's (e.g. rhesus monkey) tail is immersed in a container of warmed water hypothesized to be noxious (50 or 54 °C) and the latency for the animal to remove its tail is measured (Dykstra and Woods, 1986). Candidate analgesics or adjuncts can then be administered to determine if administration leads to an increased tail withdrawal latency indicative of an antinociceptive effect. For example, the MOR agonist morphine produces dose-dependent antinociception (Dykstra and Woods, 1986). These results and more recently published results suggest that this procedure is sensitive to clinically used opioid analgesics (Walker et al, 1993; Gatch et al, 1998; Negus et al, 2009; Banks et al, 2010a,b; Maguire and France, 2014; Cornelissen et al, 2018a,b; Cornelissen 2019). However, the warm-water tail withdrawal procedure is known to have poor behavioral selectivity and thus is susceptible to false positives due to motor suppression or incoordination (Whiteside et al, 2013; Negus, 2019).

To facilitate interpretation of results in the warm-water tail withdrawal procedure, a second behavioral procedure was utilized in this dissertation to provide one metric of behavioral selectivity. Schedule-controlled responding has been used since the early 1960's to determine pharmacological properties of drugs related to potency, time course, and receptor mechanisms (Cook and Kelleher, 1962; Kelleher and Morse, 1964). Initially, the procedure was developed to determine if some drug effects were more sensitive to positively reinforced behaviors as opposed

to negatively reinforced behaviors (i.e. shock-avoidance tasks) (Cook and Kelleher, 1962). The procedure utilizes animal subjects that have been trained to respond on a manipulandum at a predetermined (typically) ratio or interval for the presentation of a food reinforcer. Once behavior is stable, drugs can then be administered to the animal to investigate drug or dose related effects on operant rates of responding. The rate-decreasing effects of opioids in NHPs have been well characterized, and there is strong concordance between studies regarding the potency rankings for opioids to produce decreases in rates of responding (Negus et al, 1993; Butelman et al, 1996; Negus et al, 2003, Stevenson et al, 2003, 2005; Banks et al 2010a,b; Cornelissen et al, 2018a,b, 2019). Moreover, there is a strong consensus that efficacy is an important determinant in this procedure with low efficacy opioids producing limited rate suppression and high efficacy opioids producing greater to even full rate suppression (Cook and Kelleher, 1962; Byrd, 1975; Howell et al, 1988; Negus et al, 1993; Negus et al, 2003; Stevenson et al, 2005; Banks et al, 2010; Cornelissen, et al, 2018a,b; Cornelissen et al 2019). Thus, this dissertation utilized these two experimental assays (warm-water tail withdrawal and schedule-controlled responding) to address the following three specific aims:

1.) **Determining the utility of the NMDA receptor antagonists ketamine and MK-801 as candidate adjuncts to MOR agonists in the selective production of anti-allodynia using dose-addition analysis in male rhesus monkeys.**

One receptor system that might be a biological target of interest for potential adjuncts to MOR agonists in combination medication therapy is the glutamate system. The N-methyl D-aspartate (NMDA) receptor is a cation-selective ion channel found throughout the central

nervous system (CNS), which is preferentially permissible to sodium, potassium, and calcium (Vyklicky et al, 2014). The endogenous ligand is the glutamate neurotransmitter, and functional receptors are tetramers comprised of two GluN1 subunits in combination with two additional subunits: two GluN2s, two GluN3s, or one of each (Monyer et al, 1992; Ulbrich & Isacoff, 2008). Current NMDA receptor channel gating models suggest that ligand binding induces a conformational change promoting the closure of the ligand-binding domain and opening of the cation channel. Interestingly, the molecular binding requirements for NMDA receptor activation are dictated by the presence of specific subunits. To this end, GluN2 containing tetramers require binding of 2 glutamate molecules and 2 glycine co-agonist molecules (Watkins & Evans, 1981; Johnson & Ascher, 1987). GluN3 containing tetramers are activated solely upon glycine binding and are much more sparsely expressed in the CNS (Pérez-Otaño et al, 2016). Beyond this, a magnesium ion is bound in the ion channel pore when the NMDA receptor is inactive, must be displaced to allow intracellular cation flux (Furukawa et al, 2005).

Three lines of evidence support the evaluation of NMDA receptor antagonists as MOR agonist adjuncts. First, glutamate is not merely the primary excitatory neurotransmitter in the CNS, but also serves a critical role in the transmission of nociceptive impulses from the periphery to cortical regions through the spinal cord via the spinothalamic tract (Westlund et al, 1992; Meldrum, 2000). Anatomical studies have demonstrated the presence of NMDA receptors at both the spinal level within the dorsal horn and supraspinal level in nociceptive pathways (Rodriguez-Munoz et al, 2012; Bourbia et al, 2014). More specifically, immunolocalization experiments in NHPs (*Macaca fascicularis*) have indicated that nearly half of spinothalamic tract-contacting cells contained glutamatergic terminals (Westlund et al, 1992). Given the role of

glutamate in nociceptive transmission along the spinothalamic tract, we hypothesized that that inhibition of glutamate transmission should dampen nociceptive transmission.

Second, a number of NMDA receptor antagonists produce antinociception in multiple preclinical models of pain across a number of different animal species. In mice, it has been demonstrated that: (1) memantine and ketamine are antinociceptive in an assay of acetic acid-induced writhing (Malec et al, 2008) and (2) MK-801 and ACEA-1011 prevent the development of formalin-induced nociception (Vaccarino et al, 1993). Furthermore, in rats, NMDA receptor antagonists have shown antinociceptive effects at both the cellular and organismal level. Administration of the compound 7-chlorokynurenate (7CK) reduced activity (i.e. action potential firing rates) of dorsal horn C-fibers (Dickenson and Aydar, 1991) and this finding was expanded upon with ketamine and MK-801-induced antinociception in an assay of lactic acid-induced stretching (Hillhouse and Negus, 2016). Finally, the antinociceptive effects of ketamine, MK-801, phencyclidine (PCP), and dextrorphan have been demonstrated in rhesus macaques using an assay of thermal nociception (France et al, 1989). These findings have been interpreted to suggest that NMDA antagonists such as ketamine might have analgesic properties and serve as candidate MOR agonist adjuncts.

Lastly, several preclinical studies have supported the utility of NMDA receptor antagonists as adjuncts to MOR agonists across several pain states and model organisms. For example, ketamine was shown to potentiate the acute antinociceptive effects of the MOR agonists morphine, fentanyl, and sufentanil in mice, but not the antinociceptive effects of the DOR agonist SNC80 or the KOR agonist U50,488H (Baker et al, 2002). Furthermore, in rats, the active ketamine metabolites S(+)- and R(-)-norketamine enhanced morphine-induced antinociception in models of: thermal nociception, peripheral neuropathy, and inflammation

(Holtman Jr et al, 2008). Finally, MK-801, (-)-6-phosphonomethyl-decahydroisoquinoline-3-carboxylic acid (LY235959), and (+)-(1-hydroxy-3-aminopyrrolidine-2-one) ((+)-HA-966) all increased the electric shock intensity tolerated by squirrel monkeys (*Saimiri sciureus*) compared to morphine alone (Allen and Dykstra, 2001). Overall, these preclinical studies are supported by clinical evidence suggesting that although ketamine has undesirable effects, it also may serve as a useful adjunct to mu agonists (for review and recent meta-analysis, see Lee and Lee, 2016).

This dissertation evaluated NMDA antagonist and MOR agonist interactions using a preclinical capsaicin-induced thermal allodynia procedure. Allodynia is defined as the expression of a pain-related pain elicited by a stimulus that is normally innocuous and can be apparent in various clinical conditions including post-operative pain, cancer, and arthritis (Ko et al, 1998; IASP, 2017). Preclinically, allodynia can be evoked via transdermal capsaicin application (Ko et al, 1998). Capsaicin is a pungent vanilloid irritant found in chili peppers of the genus *Capsicum* and is responsible for producing their "spicy" flavor. Capsaicin binds to and activates the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor (Caterina et al, 1997). As a non-selective cation channel, TRPV1 activation increases membrane potential (Caterina et al, 1997). Transdermal capsaicin application will elicit a transient burning sensation in both humans and NHPs and results in allodynia by lowering nociceptive thresholds; thus, capsaicin application may model inflammatory clinical pain states (Simone et al, 1987, 1989; Ko et al, 1998). Ketamine has been proposed to possess anti-inflammatory properties (De Kock et al., 2013; Wang et al., 2013). Moreover, results from our own laboratory have challenged the claim that either ketamine or MOR/ketamine combinations produce antinociception against acute noxious stimuli, and opioid/NMDA antagonist interactions have not been previously assessed in preclinical allodynia models (Banks et al, 2010a). Finally, capsaicin stimulates glutamate release

from sensory afferents, and is sensitive to blockade via activation of peripheral MORs (Winter et al, 1995; Ko et al, 1998). Taken together, this background strongly supports the investigation of opioid/NMDA combinations in models of allodynia that are responsive to peripheral opioid activation. Thus, antinociceptive interactions were assessed using an assay of capsaicin-induced thermal allodynia. We hypothesized that NMDA antagonists would selectively enhance mu agonist-induced antinociception vs. mu agonist-induced rate suppression.

2.) Identifying potential pharmacological determinants of the antinociceptive interaction between the NOP receptor agonist adjunct, Ro 64-6198, and MOR agonists in male rhesus monkeys.

Another potential receptor system that might function as a useful mu opioid agonist adjunct is the nociceptin opioid peptide (NOP) system. The NOP receptor is a $G_{i/o}$-coupled GPCR and considered to be the "non-classical" fourth opioid receptor (Toll et al, 2016). The NOP receptor is activated by the endogenous opioid peptide known as nociceptin/orphanin FQ (N/OFQ) and, unlike the 3 "classical" opioid receptors, is relatively insensitive to antagonism by naltrexone (Toll et al, 2016). Moreover, a paucity of commonly employed exogenous opioid ligands are active at the NOP receptor and produce agonist-like effects except for buprenorphine, and this activity is typically only apparent at very high doses (Khroyan et al, 2009). [(1S,3aS)-8-(2,3,3a,4,5, 6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza- spiro[4.5]decan-4-one (Ro 64-6198) is a highly selective and efficacious experimental small molecule NOP receptor agonist that has contributed to our improved understanding of NOP receptor functions (Jenck et al, 2000; Dautzenberg et al, 2000). While Ro 64-6198 was initially synthesized as a potential treatment for

Alzheimer's, depression, or stress disorders, targeting these indications with NOP receptor agonists have not been successful (Jenck et al, 2000; Dautzenberg et al, 2001). Moreover, the receptors are expressed in cortical brain regions and throughout the spinal cord implicating a potential role in antinociception, anxiety, and reinforcement (Toll et al, 2016). Two lines of evidence support potential interactions between mu-opioid and NOP receptors.

First, NOP receptors are colocalized with mu-opioid receptors in both spinal and brain regions involved in nociceptive signaling pathways (for review, see Toll et al., 2016). For example, both the NOP and MOR receptor are expressed in ascending and descending pathways of the spinothalamic tract and in nociceptive processing/modulatory regions such as somatosensory cortex, thalamus, parabrachial nucleus, rostral ventral medulla, spinal cord, and within the DRG (Neal et al, 1999; Florin et al, 2000; Toll et al, 2016). More specifically, NOP receptors in the dorsal horn are mainly distributed through the deep laminae I-III, which is important for antinociception (Toll et al, 2016). Thus, due to NOP receptor localization, activation of NOP receptors would be hypothesized to produce antinociception.

Second, preclinical studies have reported species difference in the antinociceptive effects of both NOP activation by the endogenous ligand and the selective high-efficacy NOP agonist Ro 64-6198 (Jenck et al, 2000; Ko et al, 2008). Intrathecal administration of N/OFQ produced antinociceptive effects in nonhuman primates, but biphasic effects on antinociception in mice (Ko et al, 2009). Moreover, systemic Ro 64-6198 produced antinociception in rhesus monkeys (Ko et al, 2009), but not rodents (Reiss et al, 2008). Further highlighting potential species differences, systemic combinations of Ro 64-6198 and a mu-opioid agonist produced additive antinociceptive effects in mice (Reiss et al, 2008), but synergistic antinociceptive effects in rhesus monkeys (Cremeans et al, 2012).

Overall, this literature supports further evaluation of NOP and mu-opioid agonist interactions. Previous studies examining mu-opioid agonist antinociceptive interactions with other receptor systems suggest that one important determinant of these interactions may be MOR agonist efficacy (Banks et al, 2010b; Maguire and France, 2014; Negus et al, 2009). However, the degree to which mu-opioid agonist efficacy is a determinant of NOP agonist interactions is unknown. Therefore, the aim of the present study was to determine the role of mu-opioid ligand efficacy in antinociceptive interactions with the NOP agonist Ro 64-6198 in rhesus monkeys using previously described procedures (Banks et al, 2010a,b; Stevenson et al, 2003). Antinociceptive interactions between Ro 64- 6198 and six mu-opioid ligands (17-cyclopropylmethyl- 3,14β-dihyroxy-4,5α-epoxy-6α-[(3′ -isoquinolyl)acetamindo]morphinan (NAQ), buprenorphine, nalbuphine, morphine, oxycodone, and methadone) that vary in agonist-stimulated GTPγS binding from lowest to highest (Selley et al, 1998; Thompson et al, 2004; Zaidi et al, 2013) and in their in vivo effectiveness to produce antinociception (Cornelissen et al, 2018b) were investigated. For comparison, Ro 64-6198 interactions were also investigated with the selective high efficacy KOR agonist nalfurafine. Nalfurafine is not a clinically-approved analgesic and fails to produce antinociception under conditions that dissociate antinociception from behavioral sedation (Endoh et al, 2001; Lazenka et al, 2018). Drug interactions were also examined in an assay of schedule-controlled responding in a different cohort of monkeys to assess behavioral selectivity to produce antinociception vs. rate suppression. If NOP agonists are to be considered as candidate MOR agonist adjuncts, then we would hypothesize that Ro 64-6198 would robustly and selectively enhance the antinociceptive vs. rate suppressant effects of mu-opioid agonists.

3.) Expansion of the Furchgott equation for receptor theory by investigating the potential for fixed-proportion mixtures of competitive MOR antagonist and MOR agonist to manipulate antinociceptive efficacy in male rhesus monkeys.

The Furchgott equation was developed to generate theoretical curves of receptor activation level across a range of agonist doses (Ruffolo, 1982). The equation considers both agonist and antagonist values including dose, efficacy, affinity, transduction function, and receptor number to generate an "estimate" of drug effects. The Furchgott equation applies to both in vivo and in vitro procedures measuring varying levels of receptor activation. The present aim expanded the Furchgott equation to predict the in vivo effects of a fixed-proportion agonist-antagonist mixture.

Fixed-proportion mixtures have been used in both preclinical experimental models and in clinically used medications. For example, the application of dose-addition analysis to determine the relationship (e.g. subadditive, additive, supra-additive) between two drugs relies upon the generation of fixed-proportion mixtures to adequately probe a wide enough range of drug combinations to best identify an effect. Moreover, several opioid combination medications employ a fixed-proportion mixture (either with an opioid antagonist or other drug). For example, the medications suboxone(R) (buprenorphine/naloxone) and Targin(R) (oxycodone/naloxone) use a fixed-proportion approach in their dosing (Simpson et al, 2008; Yokell et al, 2011). Furthermore, Targin(R) utilizes an opioid antagonist to mitigate the undesirable MOR agonist effect constipation. This example represents one potential advantage of a fixed-proportion agonist-antagonist approach.

Pharmacodynamics is concerned with the affinity and efficacy of drugs at their receptor targets. Drug affinity can be precisely measured with ligand binding techniques, but drug efficacy to activate receptor signaling and produce downstream effects is a relative measure dependent in part on the signaling pathway(s) and downstream effects under consideration, and drug efficacies are typically described in relation to some standard high-efficacy ligand (Ruffolo, 1982; Kenakin, 2012). Although efficacy is challenging to measure, it is clearly relevant in drug development. For example, mu-opioid receptor (MOR) ligands differ in their efficacy to activate MOR-coupled signal transduction processes and produce MOR-mediated effects such as analgesia and respiratory depression. Fentanyl has high MOR efficacy and increasing fentanyl doses can produce both antinociception and lethal respiratory depression (Gerak et al., 1994; Banks et al., 2010a; Ding et al., 2016). At the other extreme of the efficacy continuum, naltrexone has little or no MOR efficacy, produces no agonist effects, and functions as a competitive reversible antagonist (Walker et al., 1994; Ko et al., 1998; Bowen et al., 2002). Between these extremes are intermediate-efficacy MOR ligands such as nalbuphine and buprenorphine, which produce submaximal stimulation of MOR signaling and a subset of agonist effects that includes analgesia but only weak respiratory depression (Gerak et al., 1994; Pitts et al., 1998; Kishioka et al., 2000). Experiments to investigate the expression and consequences of ligand efficacy at MORs or other receptor targets can be useful both to (1) determine the efficacy required to produce different effects of interest, and (2) evaluate relative efficacy of new ligands as they are developed.

One common approach to efficacy evaluations relies on the use of irreversible antagonists to evaluate the impact of reducing receptor number on expression of drug effects (Furchgott, 1966; Kenakin, 1993; Bergman et al., 2000). Efficacy requirements for different effects can be

estimated, because an irreversible antagonist will produce greater antagonism of effects with high- versus low-efficacy requirements (Zernig et al., 1997). Relative efficacies of different drugs can be estimated, because an irreversible antagonist will produce greater antagonism of a low- versus high-efficacy agonist (Zimmerman et al., 1987; Walker et al., 1998). However, studies with irreversible antagonists can be logistically challenging (e.g., due to the long duration of antagonist effects), and irreversible antagonists are not available for many receptors of interest. Decreases in receptor number can also be accomplished with genetic mutations, as in heterozygous and homozygous receptor knockout animals (Grim et al., 2016), but the degree of control over the magnitude of that decrease is limited. Receptor theory suggests an alternative, more precise, and more flexible strategy to investigate efficacy using mixtures of competitive agonists and antagonists. Figure 1 (left panel) shows a theoretical dose-effect function for a high-efficacy agonist administered alone or in the presence of increasing fixed doses of an antagonist. The familiar result is an antagonist dose-dependent rightward shift in the agonist dose-effect curve (Ko et al., 1998; Negus et al., 2003). Figure 1 (right panel) shows theoretical effects using a different experimental design, in which the agonist is administered in combination with fixed-proportional doses of the antagonist, such that increasing agonist doses are administered in combination with increasing antagonist doses. In this design, the antagonist is expected to produce proportion-dependent downward shifts in the agonist dose-effect curve, and mixtures with decreasing agonist-to-antagonist proportions have decreasing apparent efficacies to activate the receptor. This approach has two potential advantages relative to existing strategies. First, agonist-to-antagonist proportion can be precisely manipulated to yield precise increments in efficacy. Second, this approach could be applied to any receptor system for which a competitive agonist and antagonist are available.

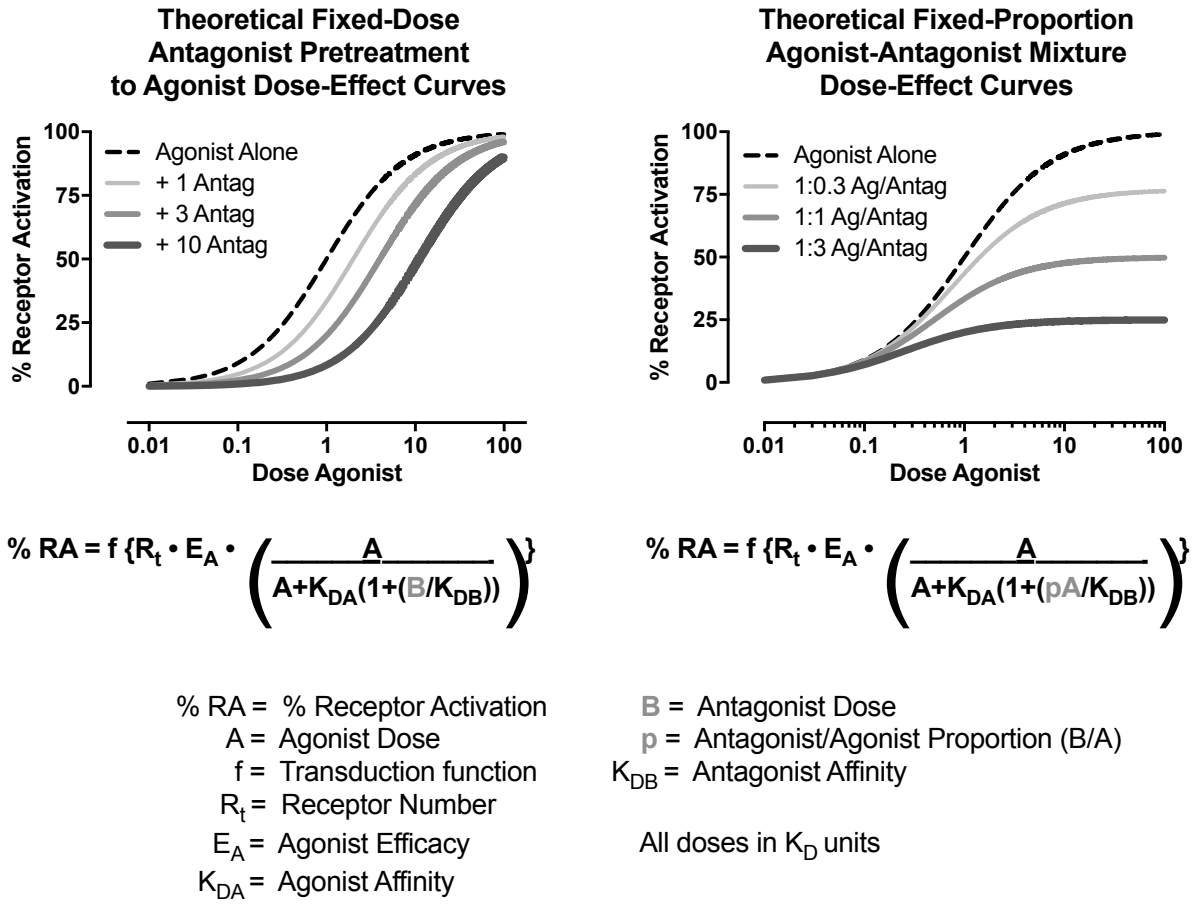


Figure 1. Theoretical curves simulated from the Furchgott equation for receptor theory (Ruffolo, 1982). Left panel shows rightward shifts in a competitive reversible agonist dose-effect function after pretreatment with increasing fixed doses of a competitive reversible antagonist. Right panel shows downward shifts in a competitive reversible agonist dose-effect function when agonist and antagonist are co-administered in fixed-proportion mixtures. Equations and definitions of terms are shown below the panels. For this simulation, agonist dose A and antagonist dose B vary in K_D (i.e., at a dose of 1, dose = K_D); R_T was set arbitrarily at 100, and all other variables were set arbitrarily at 1. Note that in the left panel, antagonist dose is a fixed proportion p of the agonist dose A, such that B = pA and increased in agonist dose are accompanied by increases in antagonist dose.

The goal of Aim 3 was to test the utility of this approach using the MOR agonist fentanyl and competitive antagonist naltrexone (Negus et al., 1993; Emmerson et al., 1994, 1996; Walker et al., 1994). Effects of these drugs administered alone and in fixed-proportion mixtures were determined in an assay of thermal nociception using two thermal stimulus intensities (50 and 54°C warm water) and compared with effects produced by six other MOR ligands shown previously to vary in their relative MOR efficacies in in vitro assays of agonist-stimulated GTPγS binding (Emmerson et al., 1996; Selley et al., 1998; Alt et al., 2001; Thompson et al.,

2004; Yuan et al., 2015). We predicted that the effects of fentanyl, naltrexone, and the mixtures would match the predicted results in Fig. 1 (right panel). Additionally, we predicted that the maximal effects of fentanyl, naltrexone, and the mixtures could be used to generate efficacy-effect scales for quantification of both (1) MOR efficacy requirements for antinociception at 50 and 54°C, and (2) relative efficacies of the six MOR test ligands.

The overarching goal of this dissertation was to utilize the opioid combination medication experimental approach in a nonhuman primate model of thermal nociception to evaluate two putative MOR agonist adjuncts. Two experimental design features were common throughout these studies. First, fixed-proportion mixtures were utilized when possible to model the clinical application of opioid adjunct combination products. Second, behavioral selectivity was assessed using an assay of schedule-controlled responding to enhance experimental rigor and provide evidence that opioid plus adjunct mixtures were not due to behavioral sedation or impairment. Our working hypothesis is that putative MOR agonist adjuncts should selectively enhance the therapeutic effects of MOR agonist compared to undesirable MOR agonist effects. Finally, the application of fixed-proportion opioid mixtures to principles of receptor theory was examined as a method to quantitatively stratify MOR agonists based on in vivo efficacy.

Chapter 2: Determining the utility of the NMDA receptor antagonists ketamine and MK-801 as candidate adjuncts to MOR agonists in the selective production of anti-allodynia using dose-addition analysis in male rhesus monkeys.

INTRODUCTION

Pain states pose a major public health challenge in the United States and around the world; one recent estimate suggest that over one-third of Americans reported pain symptoms within the past three months (Nahin, 2015). Mu-opioid agonists, such as hydrocodone and oxycodone, are increasingly being prescribed for pain management and from 2000 to 2010 there was a 4-fold increase in opioid prescriptions (Comer et al., 2013). However, mu-opioid agonists are limited in their clinical utility due to undesirable effects such as sedation and abuse liability. One drug development approach may be to combine mu agonists with adjuncts targeting other receptor systems to enhance the therapeutic effects (e.g. antinociception) and/or attenuate undesirable effects (e.g. sedation) (Dietis et al., 2009).

One receptor system that might be a biological target of interest is the glutamatergic system. Three lines of evidence support the evaluation of *N*-Methyl-*D*-Aspartate (NMDA) receptor antagonists as adjunctions to mu-opioid agonists. First, anatomical studies have demonstrated the presence of NMDA receptors at both the spinal level within the dorsal horn and supraspinal level in nociceptive pathways (Rodriguez-Munoz et al., 2012; Bourbia et al., 2014). Second, NMDA receptor antagonists, such as ketamine and dizocilpine (MK-801), produce antinociception in some, but not all, preclinical models of pain utilizing mice (Malec et al., 2008), rats (Hillhouse and Negus, 2016), and monkeys (France et al., 1989; Allen and Dykstra, 2001; Banks et al., 2010a) as research subjects. Lastly, preclinical studies in rodents have

suggested these NMDA receptor antagonists may also enhance the antiallodynic and antihyperalgesic effects mu agonists in rodents depending upon the noxious stimulus (Holtman Jr et al., 2008; Pascual et al., 2010). Furthermore, these preclinical studies are supported by some clinical evidence suggesting that although ketamine has undesirable effects, it also may serve as a useful adjunct to mu agonists under certain clinical conditions (for review and recent meta-analysis, see (McGuinness et al., 2011; Lee and Lee, 2016)). Overall, both preclinical and clinical studies support the further consideration of NMDA antagonists as adjuncts to mu agonists.

The goal of the present study was to determine whether mu-opioid agonist efficacy was a determinant of opioid/NMDA interactions in male rhesus macaques using previously described procedures for opioid interaction assessment (Stevenson et al., 2003; Banks et al., 2010a). Antiallodynic interactions were assessed using an assay of capsaicin-induced thermal allodynia for two main reasons. First, ketamine may have anti-inflammatory properties (De Kock et al., 2013; Wang et al., 2013), attenuates capsaicin-induced allodynia in humans (Park et al., 1995) and monkeys (Butelman et al., 2003), and we have previously shown that ketamine alone does not produce antinociception in monkeys using a warm water tail-withdrawal procedure (Banks et al., 2010a). Thus, one potential reason for the lack of a synergistic interaction between ketamine and fentanyl in our previous monkey study (Banks et al., 2010a) could be the preclinical nociception procedure. For example, delta-opioid agonist and mu agonist antinociceptive interactions were found to be synergistic using a strict thermal noxious stimulus (i.e. warm water tail-withdrawal), but additive under thermal allodynia (i.e. capsaicin-induced thermal allodynia) conditions (Stevenson et al., 2003; Banks et al., 2010a; Negus et al., 2012). Second, opioid/NMDA antagonist interactions have not been previously assessed in preclinical allodynia

models using nonhuman primates as research subjects and under experimental conditions using fixed-proportion mixtures and dose-addition analysis. Drug interactions were also evaluated in an assay of schedule-controlled responding for the following two reasons. First, nalbuphine, oxycodone, ketamine, and MK-801 alone produced dose-dependent effects in this procedure, and data from this procedure could be used to quantify the relative potencies of fixed-proportions in drug mixtures if one of the drugs (e.g. MK-801) was inactive in the assay of capsaicin-induced thermal allodynia. Second, drug or drug mixture effects on schedule-controlled responding provide one dependent measure of behavioral depression that may confound measures of antiallodynia in pain-stimulated behaviors (i.e. tail withdrawal). Therefore, potency comparisons of drug or drug mixture effects in assays of allodynia and schedule-controlled responding may provide an experimental index of therapeutic effect selectivity. Based on the preclinical literature, we hypothesized that NMDA antagonists would selectively enhance mu agonist-induced antiallodynia vs. mu agonist-induced rate suppression.

METHODS

Subjects:

A total of 7 adult male rhesus macaques (*Macaca mulatta*) of either Indian or Chinese origin and weighing between 10-14 kg served as subjects. Three monkeys were used in studies of schedule-controlled responding, and 4 monkeys were used in studies of capsaicin-induced thermal allodynia. All animals had prior experimental histories consisting of opioids, cocaine, and NMDA antagonist exposure. The diet consisted of laboratory monkey chow (#5049, Purina, Framingham, MA), and was supplemented daily with fresh fruits and/or nuts. Monkeys were individually housed with free access to water under a 12-h light/12-h dark cycle (lights on from

6:00 AM until 6:00 PM). The facility was licensed by the United States Department of Agriculture and accredited by AAALAC International. Both research and enrichment protocols were approved by the Institutional Animal Care and Use Committee and in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals (Council, 2011). Environmental enrichment included: music, movies, puzzle feeders, and chew toys. Furthermore, monkeys were afforded opportunities to interact socially using olfactory and auditory cues; mirrors provided visual interaction.

Behavioral Procedures

Assay of Capsaicin-Induced Thermal Allodynia

Monkeys were seated in acrylic restraint chairs as described previously (Banks et al., 2010b). The monkey's tail was shaved at least once a week 10-12 cm from the tip upward, and baseline tail-withdrawal latencies were measured from water heated to 38, 42, 46, and 50 °C. The maximal latency was 20 s, and if the monkey had not withdrawn its tail within 20 s, the experimenter removed the tail, and a latency of 20 s was assigned. Using this procedure, temperature-effect functions were determined in each monkey at the beginning of the behavioral session, and the highest temperature that failed to elicit a tail withdrawal was determined (i.e., the highest temperature to produce a tail-withdrawal latency of 20 s). Water heated to this temperature then served as the thermal stimulus for subsequent allodynia studies during that session. Allodynia was elicited by topical capsaicin application (0.3 mL of either 1.22M (n=2) or 2.44M (n=2) capsaicin) as described previously (Butelman et al., 2004; Banks et al., 2010b). After baseline tail-withdrawal latency determinations, the subject's tail was wiped with an alcohol pad and a topical capsaicin patch was prepared as described below (see Drugs); the patch

was applied to a region ~ 5 cm from the bottom of the tail for 5 min. After 5 min, the patch was removed and tail-withdrawal latencies were redetermined using the thermal stimulus identified from the baseline temperature-effect function. Initially, nalbuphine (0.032-0.32 mg/kg), oxycodone (0.01-0.32 mg/kg), ketamine (0.32-1.8 mg/kg), and MK-801 (0.0032-0.056 mg/kg) were tested alone and each dose was tested once. A single drug or drug mixture dose was administered 5 min before topical capsaicin administration and the time course of drug or drug mixture effects on tail-withdrawal latencies was determined over the course of 60 min in 15-min intervals starting 30 min post-drug or drug mixture administration. Subsequently three mixtures (1:0.33, 1:1, and 1:3 mu agonist/NMDA antagonist) of nalbuphine or oxycodone in combination with ketamine or oxycodone in combination with MK-801 were examined such that the intermediate proportion was 1:1 mu agonist/NMDA antagonist, and 3-fold lower and higher proportions were also determined. The fixed proportions for nalbuphine/ketamine and oxycodone/ketamine were based on the relative potencies of these compounds in the assay of capsaicin-induced thermal allodynia because all compounds were behaviorally active. However, fixed proportions of oxycodone/MK-801 were based on the relative potencies of these compounds in the assay of schedule-controlled responding because MK-801 did not produce > 50% maximum possible effect (MPE) in all monkeys up to doses that produced undesirable effects. Testing occurred twice weekly on Tuesdays and Fridays.

Assay of Schedule-Controlled Responding

Experiments were conducted in each monkey's housing chamber which also served as the experimental chamber as previously described (Banks et al., 2010a). A custom-fabricated operant response panel and a food pellet dispenser (Med Associates, ENV-203-1000, St. Albans,

VT) were attached to the front of the housing chamber. Panels were operated under a MED-PC interface and programmed with an IBM computer using MEDSTATE Notation (MED Associates). All behavioral training sessions were comprised of five 30-min cycles for a total session duration of 150 min. Two components were incorporated into each cycle. The first component was a 25-min time-out period during which responding was recorded, but had no scheduled consequences. The second component was a 5-min response period during which the right key was illuminated red, and subjects could respond under a fixed-ratio 30 (FR30) schedule of food pellet presentation. The response component terminated immediately and lights were extinguished if a subject earned the maximum of 10 pellets prior to completion of the 5-min period. All monkeys were trained until rates of responding were ≥ 1.0 response/s during all 5 cycles for 7 consecutive days (data not shown).

Behavioral sessions were conducted 5 days per week. Test sessions were usually conducted on Tuesdays and Fridays, and training sessions were conducted on Mondays, Wednesdays, and Thursdays. Subjects were eligible for participation in test sessions if rates of operant responding were ≥ 1.0 response/s on training days that preceded test days. On test days, test compounds were administered intramuscular (IM) using a cumulative dosing procedure, in which doses of the test drug or drug mixture were administered at the beginning of the 25-min time-out period, and each dose increased the total cumulative dose by one-fourth or one-half log units in 30-min intervals.

Initially, dose-effect functions were determined for nalbuphine (0.032-1.8 mg/kg), oxycodone (0.01- 1.0 mg/kg), ketamine (0.1-3.2 mg/kg) or MK-801 (0.0032-0.032 mg/kg) alone, and each drug was tested twice. Subsequently, three mixtures of ketamine in combination with nalbuphine or oxycodone were examined. In addition, three mixtures of MK-801 and oxycodone

were also examined. All drug mixtures were studied across a range of three fixed-proportions (1:0.33, 1:1, and 1:3 mu agonist/NMDA antagonist) such that the intermediate proportion was 1:1 mu agonist/NMDA antagonist, and 3-fold lower and higher proportions were also determined. Each mixture was tested once, and mixtures were evaluated twice a week. All drugs and drug mixtures were tested up to doses that decreased responding >50% of the preceding training day's response rate.

In addition, drug interactions can also be influence by their relative time courses. The relative time courses of ED_{80} nalbuphine, oxycodone, ketamine, and MK-801 doses were compared in the assay of schedule-controlled responding. Either saline, or a single nalbuphine (1.65 mg/kg), oxycodone (0.37 mg/kg), ketamine (1.41 mg/kg), or MK-801 (0.039 mg/kg) dose was administered, and 5 min response periods were initiated 10, 30, 100, and 300 min after drug administration.

Data Analysis

For the assay of capsaicin-induced thermal allodynia, raw tail-withdrawal latencies were converted to a percent maximum possible effect (%MPE). %MPE was defined as {(Test latency – Control latency) ÷ (20 – Control latency)*100} where "test latency" was the average latency over the 60-min test session and "control latency" was the average latency over a 60-min control session during which vehicle was administered. For the assay of schedule-controlled responding, raw rates of operant responding from each test cycle were converted to a percent control rate using the average response rate from all 5 cycles from the previous training day in that monkey.

The effective dose (ED_{50}) that produced 50%MPE or 50% decrease in control rate of responding was determined for each mu agonist alone and in combination with either ketamine

or MK-801 in each monkey for both assays. ED_{50} values were determined by interpolation when only two data points were available (one below and one above the 50% effect) or by linear regression when at least 3 data points were available on the linear portion of the dose-effect function. Individual ED_{50} values were subsequently averaged to yield mean ED_{50} values and 95% confidence limits. In addition, potency ratios were calculated for each individual subject by dividing the control ED_{50} value by the test ED_{50} value. These potency ratios were then averaged to yield group mean potency ratios and 95% confidence limits. Potency ratios were considered statistically significant if the 95% confidence limits of the group mean potency ratio did not include 1.

To evaluate drug interactions within an assay, both graphical and statistical approaches to dose-addition analysis were utilized as described previously (Stevenson et al., 2003; Banks et al., 2010a). Graphically, data for each drug and drug mixture were plotted as isobolograms at the 50% effect level. An isobologram plotted one drug dose ± SEM in a mixture as a function of the other drug dose ± SEM in a mixture at the overall mixture dose that produced 50% effect. Statistical evaluation of drug interactions was accomplished by comparing the experimentally determined ED_{50} value for each mixture (Zmix) with the predicted additivity ED_{50} value (Zadd) as previously described (Tallarida, 2000; Tallarida, 2016). Zadd values were calculated for each individual monkey using the equation: $Zadd = fA + (1 - f)B$, where A was the mu agonist alone ED_{50} value, B was the NMDA antagonist alone ED_{50} value, and f was a fractional multiplier of A in the computation of the additive total dose. The experiments described in this study tested mixtures that yielded values of f=0.25, f=0.5, and f=0.75, were f is related to the proportion of the mu agonist in a mixture per the equation ρA=f/Zadd. When mixtures were studied in the assay of capsaicin-induced thermal allodynia, where MK-801 was inactive, the

additivity hypothesis predicts the inactive drug should not contribute to the mixture effect. Thus, the equation for $Zadd = A/\rho A$. Zmix was calculated for each monkey as the total drug dose that decreased rates of responding to 50% of control or produced 50% MPE. Group mean Zmix and Zadd values were significantly different if the 95% confidence intervals did not overlap.

Drugs

(−)-Oxycodone HCl was provided by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). Racemic ketamine HCl was purchased from a commercial vendor as KetaVed© (Vedco, St. Joseph, MO). (−)-Nalbuphine HCl was provided by Dr. Kenner Rice (Drug Design and Synthesis Section, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). (+)-MK-801 hydrogen maleate was purchased from a commercial vendor (Sigma-Aldrich, St. Louis, MO). NMDA antagonists, mu-opioid agonists, and combination fixed proportions were dissolved in sterile water. Capsaicin (M2028; Sigma-Aldrich) was dissolved in a mixture of 70% ethanol (Pharmaco-AAPER, Brookfield, CT) and 30% sterile water no more than 30 minutes before use. Dissolved capsaicin was applied transdermally via an adhesive bandage measuring 2.5 x 8.3 cm (Band-Aid, Johnson and Johnson, New Brunswick, NJ). All drug doses were expressed as the salt forms listed above and administered intramuscularly into the thigh.

RESULTS

Mu-opioid agonist and ketamine interactions

Assay of capsaicin-induced thermal allodynia

The highest thermal stimulus that failed to elicit a tail-withdrawal response before capsaicin treatment was 42°C in two monkeys and 46°C in the other two monkeys throughout the study. Transdermal capsaicin application produced allodynia as indicated by reduced mean ± SEM tail withdrawal latencies at these temperatures to 2.5 ± 0.9 s, 2.0 ± 1.3 s, 2.5 ± 1.9 s, and 1.5 ± 1.0 s at 15, 30, 45, and 60 min after capsaicin treatment, respectively. Nalbuphine, oxycodone, and ketamine produced dose-dependent antiallodynia (Figure 1A). The ED_{50} values and 95% confidence limits for each drug alone are shown in Tables 1 and 2. Based on these ED_{50} values, three mixtures of nalbuphine + ketamine (1:3.3, 1:10, and 1:33 nalbuphine/ketamine) and oxycodone + ketamine (1:3.6, 1:10.7, and 1:32.1 oxycodone/ketamine) were examined. The dose ranges examined for each nalbuphine + ketamine mixture were 0.01-0.1 mg/kg nalbuphine (1:3.3), 0.01-0.1 mg/kg nalbuphine (1:10), and 0.01-0.056 mg/kg nalbuphine (1:33). The dose ranges examined for each oxycodone + ketamine mixture were 0.01-0.1 mg/kg oxycodone (1:3.6), 0.01-0.056 mg/kg oxycodone (1:10.7), and 0.0032-0.056 mg/kg oxycodone (1:32.1). Larger doses were not examined due to the emergence of undesirable effects (e.g. muscle tone loss) that impaired the monkey's ability to maintain a sufficiently sternal posture in the chair. Tables 1 and 2 also show the ED_{50} values for each drug in each mixture, and Table 3 shows the predicted Zadd and experimentally determined Zmix values for nalbuphine/ketamine and oxycodone/ketamine mixtures. The dose-effect functions for nalbuphine/ketamine and oxycodone/ketamine mixtures are shown in Figure panels 2A and 3A, respectively. Isobolograms for both drug mixtures are shown in Figure panels 2C and 3C. Combining ketamine with either nalbuphine or oxycodone did not significantly alter the potency of either mu agonist to produce antiallodynia; however, ED_{50} values were only able to be determined in 2 out of 3 monkeys with the 1:10 and 1:33 nalbuphine/ketamine mixtures and the 1:32.1

oxycodone/ketamine mixture. For nalbuphine and ketamine mixtures, the 1:3.3 and 1:10 mixtures produced additive effects. In the two monkeys that an ED_{50} value could be determined with the 1:33 nalbuphine/ketamine mixture, the effects were sub-additive. All oxycodone and ketamine mixtures produced antiallodynia effects consistent with additivity.

Assay of Schedule-Controlled Responding

The average ± SEM control response rate throughout the entire study was 2.5 ± 0.1 responses/s. Nalbuphine, oxycodone, and ketamine dose-dependently decreased rates of responding (Figure 1B). The ED_{50} values and 95% confidence limits for each drug are shown in Tables 1 and 2. Based on the relative potencies in the assay of capsaicin-induced thermal allodynia, the same three mixtures of nalbuphine + ketamine (1:3.3, 1:10, and 1:33 nalbuphine/ketamine) and oxycodone + ketamine (1:3.6, 1:10.7, and 1:32.1 oxycodone/ketamine) were examined. The dose ranges examined for each nalbuphine + ketamine mixture were 0.01-1 mg/kg nalbuphine (1:3.3), 0.01-0.32 mg/kg nalbuphine (1:10), and 0.0032-0.1 mg/kg nalbuphine (1:33). The dose ranges examined for each oxycodone + ketamine mixture were 0.01-1 mg/kg oxycodone (1:3.6), 0.01-0.32 mg/kg oxycodone (1:10.7), and 0.0032-0.1 mg/kg oxycodone (1:32.1). The dose-effect functions for nalbuphine/ketamine and oxycodone/ketamine mixtures are shown in Figure panels 2B and 3B, respectively. Isobolograms are shown in Figure panels 2D and 3D. Tables 1 and 2 also show the ED_{50} values for each drug in each mixture, and Table 3 shows the predicted Zadd and experimentally determined Zmix values for the nalbuphine/ketamine and oxycodone/ketamine mixtures, respectively. Increasing fixed proportions of ketamine enhanced the potency of nalbuphine to decrease rates of responding as demonstrated by the 95% confidence limits for the potency ratios not including

one. Dose-addition analysis demonstrated that all nalbuphine/ketamine mixtures produced additive effects. For oxycodone and ketamine mixtures, fixed proportions of 1:10.7 and 1:32.1 increased the potency of oxycodone to decrease rates of responding compared to oxycodone alone. Dose-addition analysis demonstrated the 1:3.6 and 1:10.7 oxycodone/ketamine mixtures produced sub-additive effects; whereas the 1:32.1 oxycodone/ketamine mixture produced additive effects.

Oxycodone and MK-801 interactions

Assay of capsaicin-induced thermal allodynia

Oxycodone alone produced dose-dependent antiallodynia, whereas MK-801 produced a maximum %MPE of 22.7 ± 17.8 at a dose of 0.032 mg/kg (Figure 1A). The ED_{50} values and 95% confidence limits for oxycodone alone and each drug in the drug mixture are shown in Table 4. Because MK-801 produced > 50%MPE in only one subject, the relative potencies were based on the ED_{50} values in the assay of schedule-controlled responding (below). The dose ranges examined for all oxycodone + MK-801 mixtures were 0.01-0.32 mg/kg oxycodone and larger doses were not examined due to the emergence of undesirable effects (e.g. muscle tone loss) that impaired the monkey's ability to maintain a sufficiently sternal posture in the chair. The dose-effect functions for oxycodone/MK-801 mixtures are shown in Figure panels 4A. The isobologram for the three oxycodone + MK-801 mixtures are shown in Figure 4C. Increasing fixed proportions of MK-801 did not significantly alter the potency of oxycodone to produce antiallodynia (Table 4). Table 5 shows the predicted Zadd and experimentally determined Zmix values for the oxycodone/MK-801 mixtures. The 1:0.028 and 1:0.085 oxycodone/MK-801

mixture produced effects consistent with additivity, whereas the 1:0.25 oxycodone/MK-801 mixtures produced significant sub-additive effects.

Assay of Schedule-Controlled Responding

Oxycodone and MK-801 alone produced dose-dependent decreases in rates of responding (Figure 1B). The ED_{50} values and 95% confidence limits for each drug are shown in Table 4, and based on the relative potencies, three mixtures of oxycodone + MK-801 (1:0.028, 1:0.085, and 1:0.25 oxycodone/MK-801) were examined. The dose ranges examined for each oxycodone + MK-801 mixture were 0.01-1 mg/kg oxycodone (1:0.028), 0.01-0.56 mg/kg oxycodone (1:0.085), and 0.01-0.32 mg/kg oxycodone (1:0.25). The dose-effect functions for oxycodone/MK-801 mixtures are shown in Figure panel 4B. The isobologram for all three oxycodone + MK-801 mixtures are shown in Figure 4D. Fixed proportions (0.028 and 0.25) of MK-801 significantly enhanced the potency of oxycodone to decrease rates of responding (Table 4). Table 5 shows the predicted Zadd and experimentally determined Zmix values for each drug mixture. All oxycodone and MK-801 mixtures produced effects consistent with additivity.

Time Course Analysis

Figure 5 shows the time course of nalbuphine, oxycodone, ketamine, and MK-801 in the assay of schedule-controlled responding. Two-way repeated-measures analysis of variance demonstrated a significant main effect of time ($F_{3,6}$=164.9, $p<0.0001$), drug ($F_{4,8}$=28.9, $p<0.0001$), and drug × time interaction ($F_{12,24}$=8.5, $p<0.0001$). All four drugs produced significant and peak rate-decreasing effects within 10-30 min post administration. MK-801 did produce rate-decreasing effects that were significantly different from oxycodone and ketamine,

but not nalbuphine, at the 30-min time point. Nalbuphine and MK-801 produced rate-decreasing effects that persisted to at least 100 min and were significantly longer than oxycodone and ketamine.

DISCUSSION

This study assessed interactions between the noncompetitive NMDA antagonists racemic ketamine and (+)-MK-801 and the low-efficacy mu agonist nalbuphine and the moderate-efficacy mu agonist oxycodone in assays of capsaicin-induced thermal allodynia and schedule-controlled responding in rhesus monkeys. The main finding was that both racemic ketamine and (+)-MK-801 failed to enhance the antiallodynic effects of nalbuphine and oxycodone. Furthermore, ketamine selectively enhanced the potency of both nalbuphine and oxycodone to produce rate suppression. Overall, these preclinical results in monkeys do not support further consideration of noncompetitive NMDA antagonists as clinically useful adjuncts to mu-opioid agonists for the treatment of pain states associated with thermal hypersensitivity.

Effects of Mu agonists and NMDA antagonists alone

Consistent with previous studies in rodents (Emery et al., 2017), nonhuman primates (Banks et al., 2010b; Negus et al., 2012), and humans (Watson and Babul, 1998; Hoeben et al., 2012), both nalbuphine and oxycodone produced dose-dependent antiallodynia. Both nalbuphine and oxycodone dose-dependently decreased rates of operant responding and these nalbuphine results were consistent with previous nonhuman primate studies examining the rate-suppressant effects of mu-opioid agonists (Stevenson et al., 2003; Banks et al., 2010a). Furthermore, the present results extended these previous findings by determining the antiallodynic and rate-

suppressant effects of the mu agonist oxycodone in nonhuman primates. Oxycodone was approximately 4 to 5-fold more potent to produce antiallodynia vs. rate suppression.

Although both ketamine and MK-801 produced dose-dependent decreases in rates of responding, only ketamine produced dose-dependent antiallodynia in the assay of capsaicin-induced thermal allodynia. Both the rate-suppressant and antiallodynia effects of ketamine in the present study are consistent with previous results in rhesus monkeys (Butelman et al., 2003; Banks et al., 2010a). Furthermore, the present MK-801 results are consistent with a previous study in mice (Gewehr et al., 2011). In apparent contrast to the present results demonstrating MK-801 failed to produce antiallodynia, previous nonhuman primate studies have reported antiallodynic effects of MK-801 in an assay of capsaicin-induced thermal allodynia (Butelman et al., 2003) and antinociceptive effects in an assay of thermal nociception (France et al., 1989). However, the anti-allodynic effects of MK-801 were only present at a low thermal stimulus of 38°C and MK-801 antiallodynic effects were more variable when the thermal stimulus was increased to 42°C (Butelman et al., 2003). In the present study, the baseline thermal intensities before capsaicin application were 42°C for two monkeys and 46°C for the other two monkeys. In the one monkey that did show an antiallodynic effect of MK-801, the baseline thermal intensity was 42°C. Another explanation for the differential ketamine and MK-801 results in the assay of capsaicin-induced thermal allodynia could be due to ketamine interacting with other receptor systems than NMDA. For example, racemic ketamine has been shown to have similar affinity for opioid receptors compared to the NMDA channel binding site (Hustveit et al., 1995). Overall, the general consistency of the present results with the published literature provided an empirical foundation to determine mu agonist and NMDA antagonist interactions.

Antiallodynia interactions

No fixed proportion of either NMDA antagonist selectively enhanced the antiallodynic effects of nalbuphine or oxycodone. The present additive antiallodynic results are consistent with ketamine and alfentanil analgesic interactions in humans using intradermal capsaicin (Sethna et al., 1998). Furthermore, the present additive or sub-additive interactions are consistent with and extend previous findings from our laboratory (Banks et al., 2010a) and others (Hoffmann et al., 2003; Craft and Lee, 2005; Edwards et al., 2007; Haghparast et al., 2007; Pascual et al., 2010; Lilius et al., 2015) examining NMDA antagonist and mu-opioid agonist combinations in other preclinical assays of nociception, including allodynia. However, the present results may appear in contrast to previous studies in rodents demonstrating that NMDA antagonists enhance the antiallodynic/antihyperalgesic effects of mu agonists (Holtman Jr et al., 2008; Pascual et al., 2010). There are two potential explanations for the differential results between the present study and these previous studies reporting an enhanced antinociceptive effect of the mu agonist.

First, drug interactions are not only dependent upon the relative dose, but also time course of each drug in a mixture. Thus, differences in mu agonist and NMDA antagonist time course could have influenced drug interactions when conducting fixed proportion interaction studies. This explanation seems unlikely for the following two reasons. First, peak rate decreasing effects were at 10 min for nalbuphine, oxycodone, and ketamine and 30 min for MK-801 in the assay of schedule-controlled responding (Figure 5). Furthermore, all drugs produced significant rate-decreasing effects for at least 30 min, which was within the pretreatment time range used in assay of schedule-controlled responding. Second, statistical analyses did not demonstrate a significant main effect of time in the assay of capsaicin-induced thermal allodynia for any drug alone or drug mixture. Overall, these results suggest that modest differences in time

course between the drugs do not fully explain the absence of a selective NMDA antagonist enhancement of mu agonist antiallodynia.

Second, behavioral selectivity to produce antiallodynia vs. suppression of operant responding may also explain the differential results. Pain-stimulated behaviors, such as tail withdrawal, are highly susceptible to false positive results due to drug-induced motor impairment. In paclitaxel-treated rats, ketamine and morphine combinations produced an enhanced anti-thermal hyperalgesic effect, but no interaction on mechanical allodynia and no assessment of motor activity (Pascual et al., 2010). In a rat chronic constriction nerve injury model, the ketamine metabolite norketamine enhanced the antiallodynic and antihyperalgesic effects of morphine at dose combinations that did not significantly alter behavior in a rotorod or locomotor activity procedure (Holtman Jr et al., 2008). Moreover, a recent meta-analysis of ketamine and opioid use for pain reduction found that ketamine did not generally enhance pain relief produced by opioids (only 1 out of 6 showed an enhancement) and in fact, may enhance some undesirable neurological and psychological undesirable effects (Lee and Lee, 2016). Overall, the literature suggests that NMDA antagonists may selectively enhance the antiallodynic/antihyperalgesic effects of mu agonists over a narrow range of experimental conditions that depend upon not only the research subject species, but also the type of noxious stimulus and underlying physiological state.

Comparison with other mu agonist interactions

The present mu agonist and NMDA antagonist interaction results can be compared to results that have determined fixed-proportion drug mixtures of other drug classes in combination with mu agonists under similar procedures. Two will be mentioned. First, the serotonin and

norepinephrine uptake inhibitor clomipramine selectively enhanced both the antiallodynic and antinociceptive vs. rate-suppressant effects of nalbuphine in monkeys (Banks et al., 2010b). Second, the delta-opioid agonist SNC80 has also selectively enhanced the antiallodynic and antinociceptive vs. rate-suppressant effects of nalbuphine in monkeys (Stevenson et al., 2003; Negus et al., 2012). In contrast, fixed proportions of NMDA antagonists and mu agonists have thus far failed to produce a selective enhancement of antinociception vs. rate suppression in both assays of capsaicin-induced thermal allodynia (present results) and warm water tail withdrawal (Banks et al., 2010a) and. Overall, this literature highlights the utility of the behavioral procedures described in the present study to examine mu agonist and other drug class interactions in the development of mu opioid adjuncts for the clinical treatment of various pain states.

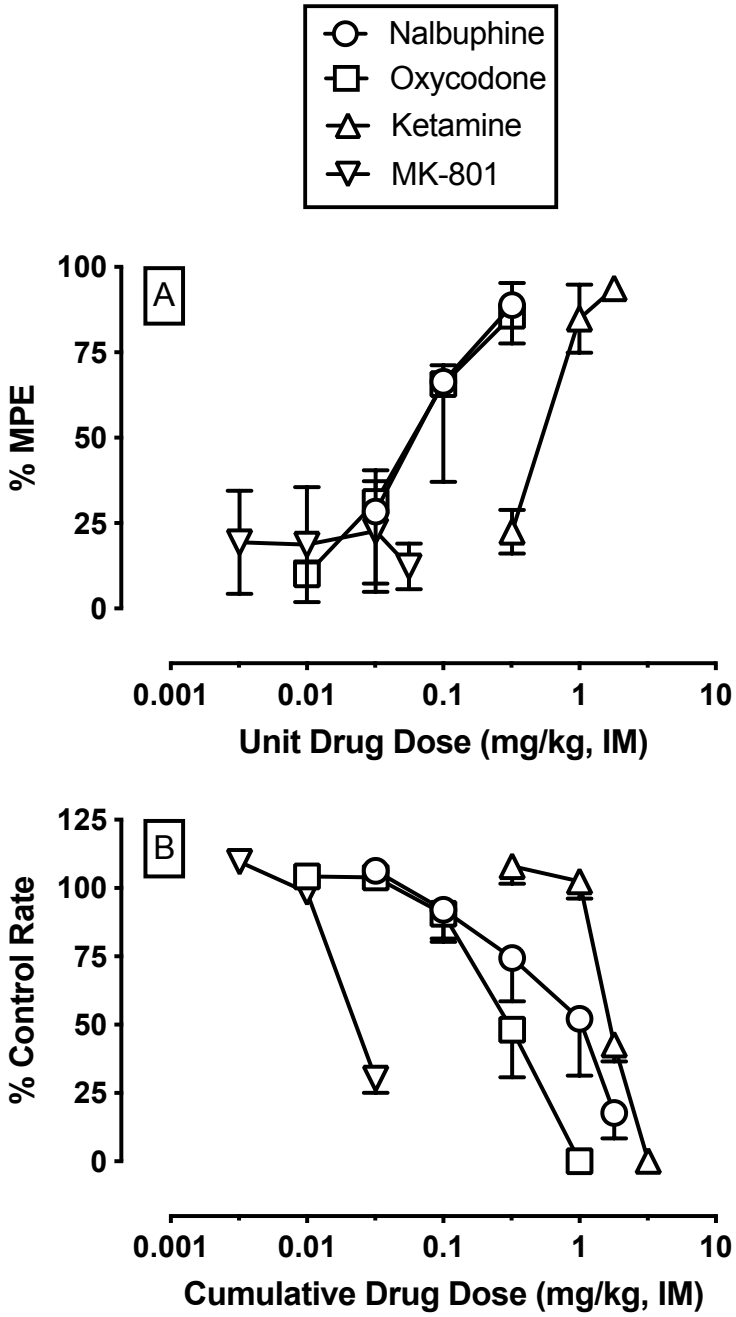


Figure 1

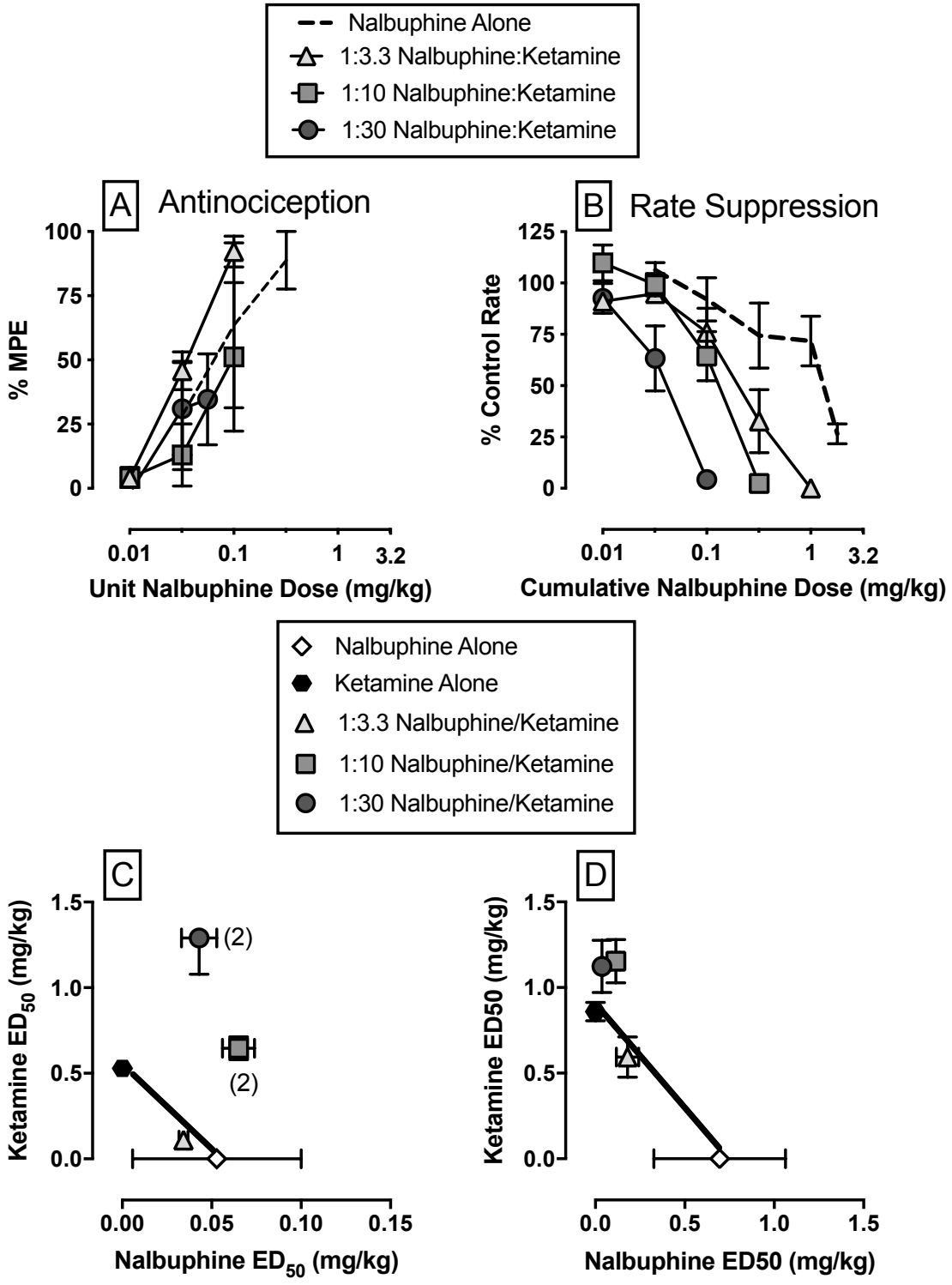


Figure 2

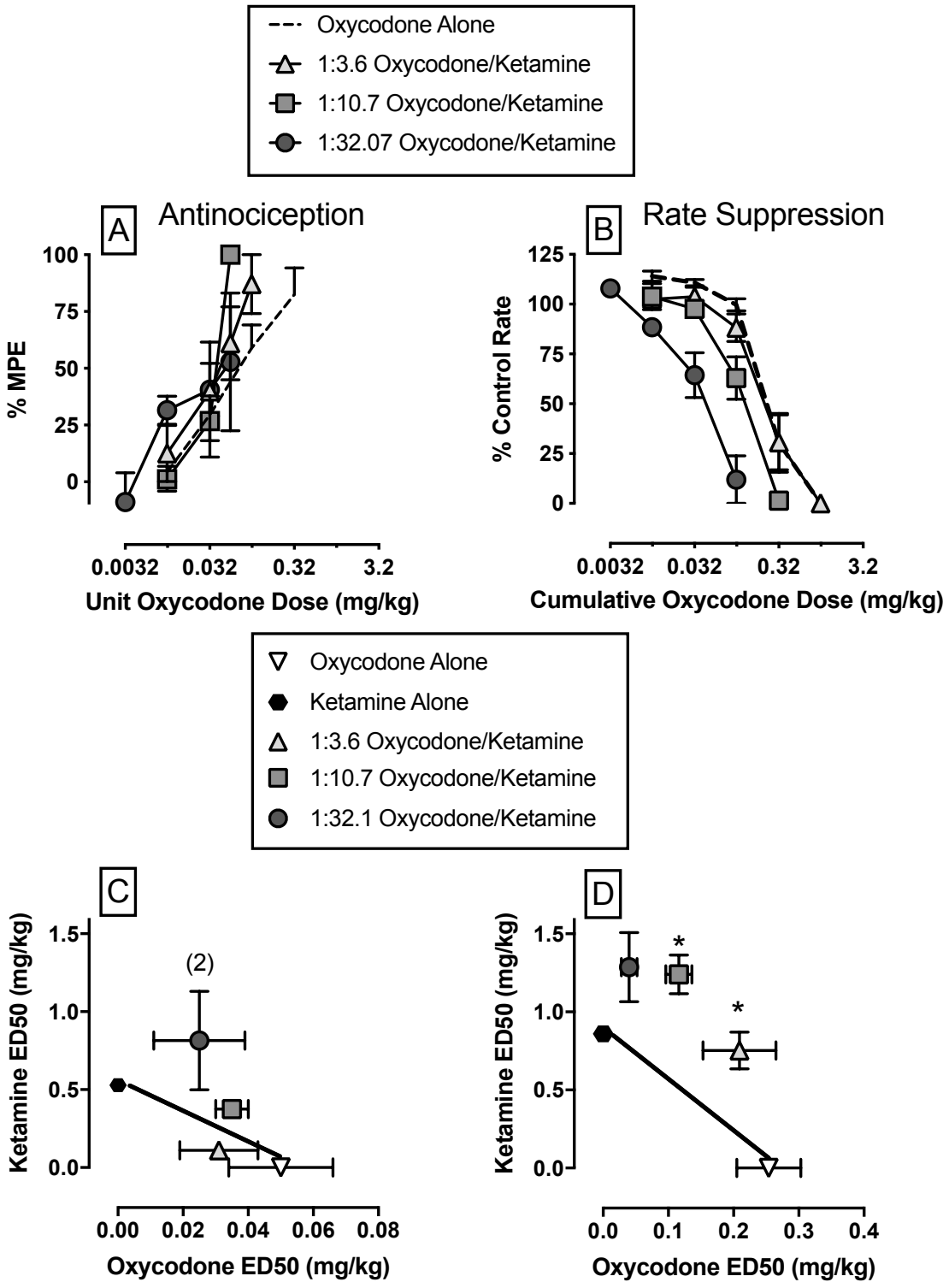


Figure 3

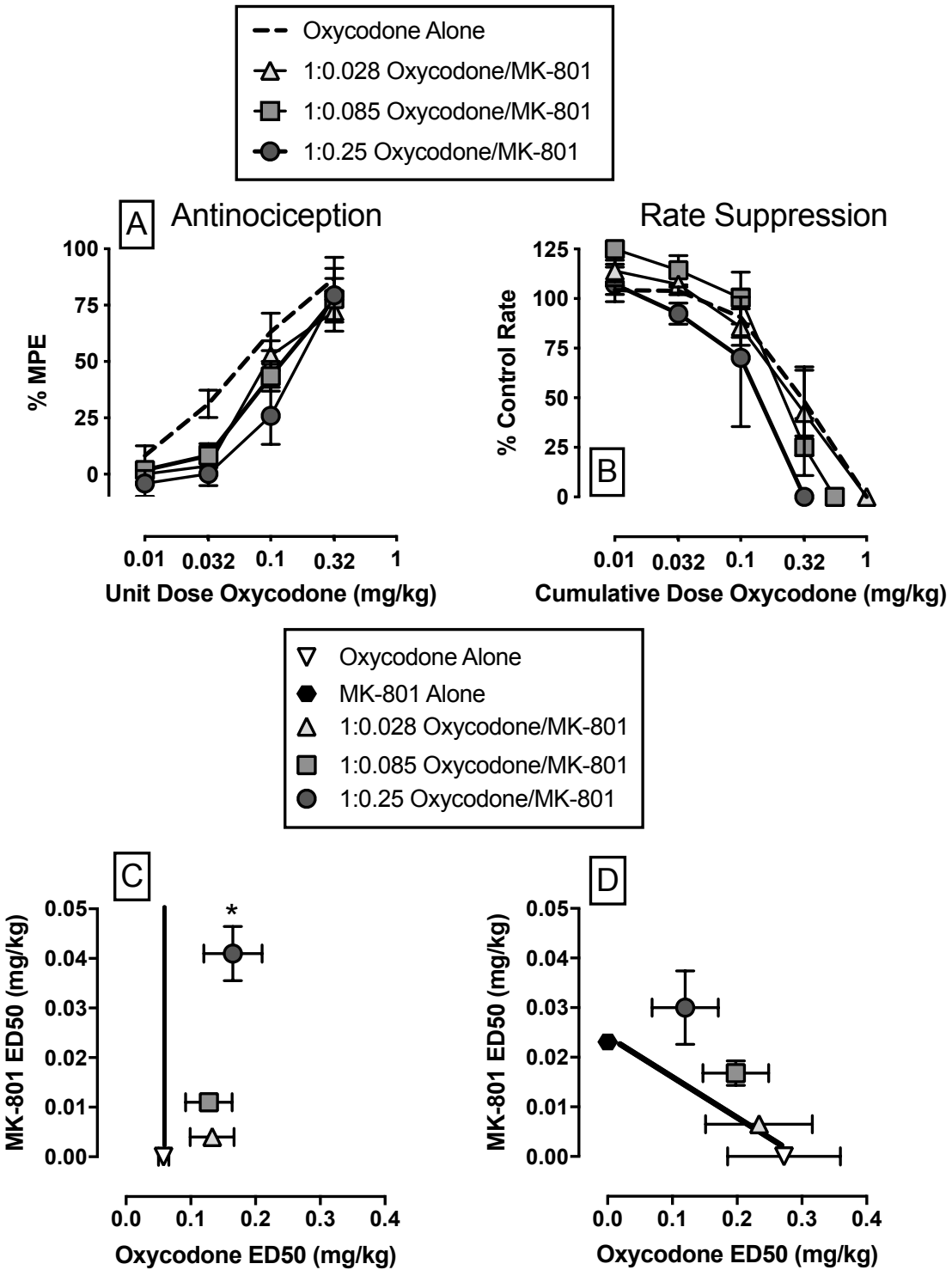


Figure 4

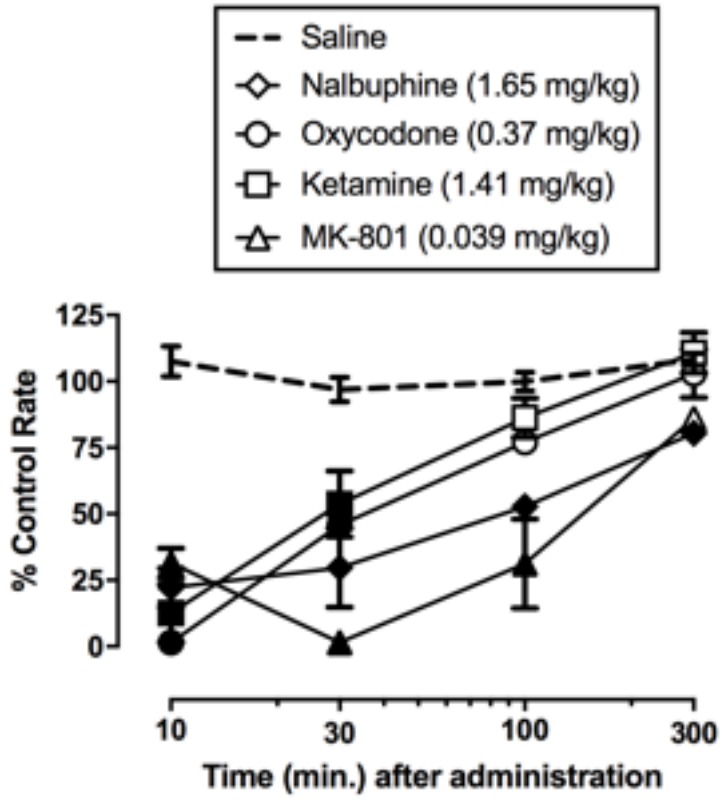


Figure 5

FIGURE LEGENDS

Figure 1: Potency of nalbuphine, oxycodone, ketamine, and MK-801 to produce anti-allodynia in an assay of capsaicin-induced thermal allodynia (Panel A; n=3-4) and decrease rates of responding in an assay of schedule-controlled responding (Panel B; n=3) in rhesus monkeys. Top abscissae: unit intramuscular (IM) drug dose in milligrams per kilogram (log scale). Top ordinate: percent maximum possible effect. Bottom abscissae: cumulative intramuscular (IM) drug dose in milligrams per kilogram (log scale). Bottom ordinate: percent control rate of responding. Each point shows mean ± SEM for 3-4 monkeys.

Figure 2: Effects of the mu-opioid agonist nalbuphine alone or in combination with the noncompetitive NMDA antagonist ketamine on capsaicin-induced thermal allodynia (left panels) and rates of schedule-controlled responding (right panels). Top panels show dose-effect functions for nalbuphine alone or in combination with ketamine and bottom panels show isobolograms at the ED_{50} effect level for nalbuphine or ketamine alone or as part of a mixture. Top abscissae: unit nalbuphine dose (left panel) or cumulative nalbuphine dose (right panel) in milligrams per kilogram per injection. Top ordinates: percent control rate of responding. Bottom panels show isobolograms at the ED_{50} effect level for nalbuphine or ketamine alone or as part of a mixture. Bottom abscissae: ED_{50} values for nalbuphine alone or in a mixture in milligrams per kilogram (linear scale). Bottom ordinates: ED_{50} values for ketamine alone or in a mixture in milligrams per kilogram (linear scale). Each point represents mean ± SEM of 3-4 monkeys, except when noted by the number in parentheses. This denotes an experimental condition where an ED_{50} value could not be determined in all subjects tested.

Figure 3: Effects of the mu-opioid agonist oxycodone alone or in combination with the noncompetitive NMDA antagonist ketamine on capsaicin-induced thermal allodynia (left panels) and rates of schedule-controlled responding (right panels). Top panels show dose-effect functions for oxycodone alone or in combination with ketamine and bottom panels show isobolograms at the ED_{50} effect level for oxycodone or ketamine alone or as part of a mixture. Top abscissae: unit oxycodone dose (left panel) or cumulative oxycodone dose (right panel) in milligrams per kilogram per injection. Top ordinates: percent control rate of responding. Bottom panels show isobolograms at the ED_{50} effect level for oxycodone or ketamine alone or as part of a mixture. Bottom abscissae: ED_{50} values for oxycodone alone or in a mixture in milligrams per kilogram (linear scale). Bottom ordinates: ED_{50} values for ketamine alone or in a mixture in milligrams per kilogram (linear scale). Each point represents mean ± SEM of 3-4 monkeys. Asterisk indicates that the mixture produced a sub-additive effect in the assay of schedule-controlled responding as determined by dose-addition analysis (see Table 3).

Figure 4: Effects of the mu-opioid agonist oxycodone alone or in combination with the noncompetitive NMDA antagonist MK-801 on capsaicin-induced thermal allodynia (left panels) and rates of schedule-controlled responding (right panels). Top panels show dose-effect functions for oxycodone alone or in combination with MK-801 and bottom panels show isobolograms at the ED_{50} effect level for oxycodone or MK-801 alone or as part of a mixture. Top abscissae: unit oxycodone dose (left panel) or cumulative oxycodone dose (right panel) in milligrams per kilogram per injection. Top ordinates: percent control rate of responding. Bottom abscissae: ED_{50} values for oxycodone alone or in a mixture in milligrams per kilogram (linear scale). Bottom ordinates: ED_{50} values for MK-801 alone or in a mixture in milligrams per

kilogram (linear scale). Each point represents mean ± SEM of 3-4 monkeys. Asterisk indicates that the mixture produced a sub-additive effect in the assay of capsaicin-induced thermal allodynia as determined by dose-addition analysis (see Table 5).

Figure 5: Time course of ED_{80} doses of nalbuphine (1.65 mg/kg), oxycodone (0.37 mg/kg), ketamine (1.41 mg/kg), and MK-801 (0.039 mg/kg) in an assay of schedule controlled responding in rhesus monkeys. Abscissa: time in min. post administration. Ordinate: percent control rate of responding. Each point represents the mean ± SEM of three rhesus monkeys. Filled symbols denote statistical significance ($p<0.05$) compared to vehicle.

Table 1: ED_{50} values (95% confidence limits) for nalbuphine, oxycodone, and ketamine alone or in combination in assays of capsaicin-induced thermal allodynia and scheduled-controlled responding in rhesus monkeys. Numbers in parentheses denote the number of subjects contributing to the ED_{50} value if less than the total number of monkeys tested (n=3) and the > symbol denotes a drug mixture for which an ED_{50} value could not be determined in all subjects tested.

Drug or drug mixture	**Nalbuphine ED_{50} (95% CL)**	**Potency Ratio (95% CL)**	**Ketamine ED_{50} (95% CL)**
Capsaicin-induced thermal allodynia			
Nalbuphine Alone	0.05 (0, 0.36)		-
Ketamine Alone	-		0.53 (0.31, 0.91)
1:3.3 Nalbuphine/Ketamine	0.03 (0.02, 0.04)	1.38 (-2.91, 5.66)	0.11 (0.08, 0.15)
1:10 Nalbuphine/Ketamine (n=2)	> 0.06 (0.03, 0.1)		> 0.65 (0.1, 4.15)
1:33 Nalbuphine/Ketamine (n=2)	> 0.04 (0, 0.17)		> 1.29 (0.07, 24.12)
Schedule-controlled responding			
Nalbuphine Alone	0.7 (0.25, 1.96)		-
Ketamine Alone	-		0.86 (0.70, 1.06)
1:3.3 Nalbuphine/Ketamine	0.18 (0.09, 0.35)	0.28 (-0.06, 0.63)[a]	0.59 (0.30, 1.16)
1:10 Nalbuphine/Ketamine	0.12 (0.08, 0.17)	0.21 (-0.24,	1.15 (0.80, 1.67)

		0.66)[a]	
1:33 Nalbuphine/Ketamine	0.04 (0.02, 0.06)	0.07 (-0.07, 0.20)[a]	1.12 (0.71, 1.79)

[a] Potency shifts were considered statistically significant if the 95% confidence limits of the potency ratios did not include 1.

Table 2: ED_{50} values (95% confidence limits) for oxycodone and ketamine alone or in combination assays of capsaicin-induced thermal allodynia and scheduled-controlled responding and in rhesus monkeys. Numbers in parentheses denote the number of subjects contributing to the Zmix or Zadd values if less than the total number of monkeys tested (n=3) and the > symbol denotes a drug mixture for which an ED_{50} value could not be determined in all subjects tested.

Drug or drug mixture	**Oxycodone ED_{50} (95% CL)**	**Potency Ratio (95% CL)**	**Ketamine ED_{50} (95% CL)**
Capsaicin-induced thermal allodynia			
Oxycodone Alone	0.05 (0.01, 0.2)		-
Ketamine Alone	-		0.53 (0.31, 0.91)
1:3.6 Oxycodone/Ketamine	0.03 (0.01, 0.17)	0.61 (-0.56, 1.78)	0.11 (0.05, 0.25)
1:10.7 Oxycodone/Ketamine	0.04 (0.02, 0.07)	0.56 (0.11, 1.02)	0.38 (0.28, 0.51)
1:32.1 Oxycodone/Ketamine (n=2)	> 0.03 (0, 0.21)		> 0.82 (0.28, 2.39)
Schedule-controlled responding			
Oxycodone Alone	0.25 (0.17, 0.37)		-
Ketamine Alone	-		0.86 (0.70, 1.06)
1:3.6 Oxycodone/Ketamine	0.21 (0.12, 0.36)	0.83 (0.44, 1.23)	0.75 (0.44, 1.28)
1:10.7 Oxycodone/Ketamine	0.12 (0.08, 0.16)	0.47 (0.17, 0.76) [a]	1.24 (0.88, 1.75)
1:32.1 Oxycodone/Ketamine	0.04 (0.02, 0.07)	0.17 (0, 0.34) [a]	1.29 (0.72, 2.31)

[a] Potency shifts were considered statistically significant if the 95% confidence limits of the potency ratios did not include 1.

Table 3: Experimentally determined Zmix values and predicted Zadd values (95% confidence limits) for mixtures of nalbuphine or oxycodone and ketamine in assays of capsaicin-induced thermal allodynia and schedule-controlled responding in rhesus monkeys. Numbers in parentheses denote the number of subjects contributing to the Zmix or Zadd values if less than the total number of monkeys tested (n=3) and denote a drug mixture for which an ED_{50} value could not be determined.

Drug or drug mixture	**Zmix**	**Zadd**
Capsaicin-induced thermal allodynia		
1:3.3 Nalbuphine/Ketamine	0.15 (0.13, 0.17)	0.19 (0.11, 0.34)
1:10 Nalbuphine/Ketamine (n=2)	0.71 (0.53, 0.95)	0.33 (0.18, 0.61)
1:33 Nalbuphine Ketamine (n=2)	1.33 (0.85, 2.1) [a]	0.43 (0.26, 0.71)
1:3.6 Oxycodone/Ketamine	0.15 (0.07, 0.32)	0.17 (0.15, 0.20)
1:10.7 Oxycodone/Ketamine	0.41 (0.31, 0.55)	0.25 (0.18, 0.35)
1:32.1 Oxycodone/Ketamine (n=2)	0.84 (0.29, 2.45)	0.41 (0.28, 0.61)
Schedule-controlled responding		
1:3.3 Nalbuphine/Ketamine	0.77 (0.40, 1.51)	0.80 (0.45, 1.45)
1:10 Nalbuphine/Ketamine	1.27 (0.88, 1.84)	0.85 (0.64, 1.14)
1:33 Nalbuphine/Ketamine	1.61 (0.73, 1.85)	0.87 (0.79, 0.95)
1:3.6 Oxycodone/Ketamine	0.96 (0.57, 1.63) [a]	0.42 (0.39, 0.44)
1:10.7 Oxycodone/Ketamine	1.36 (0.96, 1.91) [a]	0.57 (0.52, 0.62)
1:32.1 Oxycodone/Ketamine	1.33 (0.74, 2.38)	0.71 (0.61, 0.84)

[a] Denotes Zmix confidence limits do not overlap with Zadd confidence limits: Zmix larger than Zadd indicating sub-additivity.

Table 4: ED_{50} values (95% confidence limits) for oxycodone and MK-801 alone or in combination assays of scheduled-controlled responding and capsaicin-induced thermal allodynia in rhesus monkeys.

Drug or drug mixture	**Oxycodone ED_{50} (95% CL)**	**Potency Ratio (95% CL)**	**MK-801 ED_{50} (95% CL)**
Capsaicin-induced thermal allodynia			
Oxycodone Alone	0.05 (0.04, 0.06)		-
MK-801 Alone	-		Inactive
1:0.028 Oxycodone/MK-801	0.13 (0.06, 0.3)	2.64 (-1.69, 6.98)	0.004 (0.002, 0.008)
1:0.085 Oxycodone/MK-801	0.13 (0.05, 0.32)	2.09 (-2.71, 6.9)	0.011 (0.004, 0.027)
1:0.25 Oxycodone/MK-801	0.17 (0.07, 0.4)	2.37 (-2.12, 6.87)	0.041 (0.017, 0.099)
Schedule-controlled responding			
Oxycodone Alone	0.27 (0.15, 0.51)		-
MK-801 Alone	-		0.02 (0.02, 0.03)
1:0.028 Oxycodone/MK-801	0.23 (0.12, 0.47)	0.86 (0.75, 0.98) [a]	0.01 (0.003, 0.013)
1:0.085 Oxycodone/MK-801	0.20 (0.12, 0.33)	0.74 (0.35, 1.14)	0.02 (0.01, 0.03)
1:0.25 Oxycodone/MK-801	0.12 (0.05, 0.28)	0.45 (0.19,	0.03 (0.01, 0.07)

		0.71) [a]	

[a] Potency shifts were considered statistically significant if the 95% confidence limits of the potency ratios did not include 1.

Table 5: Experimentally determined Zmix values and predicted Zadd values (95% confidence limits) for mixtures of oxycodone and MK-801 in assays of capsaicin-induced thermal allodynia and schedule-controlled responding in rhesus monkeys.

Drug or drug mixture	**Zmix**	**Zadd**
Capsaicin-induced thermal allodynia		
1:0.028 Oxycodone/MK-801	0.14 (0.08, 0.23)	0.06 (0.05, 0.08)
1:0.085 Oxycodone/MK-801	0.14 (0.08, 0.24)	0.06 (0.05, 0.08)
1:0.25 Oxycodone/MK-801	0.21 (0.12, 0.35) [a]	0.07 (0.06, 0.09)
Schedule-controlled responding		
1:0.028 Oxycodone/MK-801	0.24 (0.12, 0.48)	0.21 (0.12, 0.39)
1:0.085 Oxycodone/MK-801	0.22 (0.13, 0.36)	0.15 (0.09, 0.26)
1:0.25 oxycodone/MK-801	0.15 (0.07, 0.35)	0.09 (0.06, 0.14)

[a] Denotes Zmix confidence limits do not overlap with Zadd confidence limits: Zmix larger than Zadd indicating sub-additivity.

Chapter 3: Identifying potential pharmacological determinants of the antinociceptive interaction between the NOP receptor agonist adjunct, Ro 64-6198, and MOR agonists in male rhesus monkeys.

1. INTRODUCTION

Pain management remains a significant public health issue in the United States (Ballantyne et al, 2017). Mu-opioid receptor agonists (e.g. oxycodone) are one of the most effective pharmacological tools available to clinicians for the treatment of pain (Dowell and Haegerich, 2016). However, the clinical utility of mu-opioid agonists is severely limited by a number of undesirable effects including respiratory depression and sedation. One approach to enhance the clinical utility of mu-opioid agonists may be to combine them with other compounds that act through different receptor mechanisms (Di Cesare Mannelli et al., 2015; Dietis et al., 2009; Gunther et al., 2017).

One potential receptor system that might function as a useful mu-opioid agonist adjunct is the nociceptin opioid peptide (NOP) system. Two lines of evidence support potential interactions between mu-opioid and NOP receptors. First, NOP receptors are colocalized with mu-opioid receptors in both spinal and brain regions involved in nociceptive signaling pathways (for review, see Toll et al. 2016). Second, preclinical studies have reported species difference in the antinociceptive effects of the selective high-efficacy NOP agonist Ro 64-6198 (Jenck et al., 2000). For example, systemic Ro 64-6198 produced antinociception in monkeys (Ko et al., 2009), but not rodents (Reiss et al., 2008). Further highlighting potential species differences, systemic combinations of Ro 64-6198 and a mu-opioid agonist produced additive antinociceptive effects in mice (Reiss et al., 2008), but synergistic antinociceptive effects in rhesus monkeys

(Cremeans et al., 2012). Overall, this literature supports further evaluation of NOP and mu-opioid agonist interactions.

Previous studies examining mu-opioid agonist antinociceptive interactions with other receptor systems suggest that one important determinant of these interactions may be MOR agonist efficacy (Banks et al., 2010b; Maguire and France, 2014; Negus et al., 2009). However, the degree to which mu-opioid agonist efficacy is a determinant of NOP agonist interactions is unknown. Therefore, the aim of the present study was to determine the role of mu-opioid ligand efficacy in antinociceptive interactions with the NOP agonist Ro 64-6198 in rhesus monkeys using previously described procedures (Banks et al., 2010b; Stevenson et al., 2003). Antinociceptive interactions between Ro 64-6198 and six mu-opioid ligands (17-cyclopropylmethyl- 3,14β-dihyroxy-4,5α-epoxy-6α-[(3′-isoquinolyl)acetamindo]morphinan (NAQ), buprenorphine, nalbuphine, morphine, oxycodone, and methadone) that vary in agonist-stimulated GTPγS binding from lowest to highest (Selley et al., 1998; Thompson et al., 2004; Zaidi et al., 2013) and in their in vivo effectiveness to produce antinociception (Cornelissen et al., 2018a) were investigated. For comparison, Ro 64-6198 interactions were also investigated with the selective high efficacy kappa-opioid receptor agonist nalfurafine. Nalfurafine is not a clinically-approved analgesic and fails to produce antinociception under conditions that dissociate antinociception from behavioral sedation (Endoh et al., 2001; Lazenka et al., 2018). Drug interactions were also examined in an assay of schedule-controlled responding in a different cohort of monkeys to assess behavioral selectivity to produce antinociception vs. rate suppression. If NOP agonists are to be considered as candidate mu-opioid agonist adjuncts, then we would hypothesize that Ro 64-6198 would robustly and selectively enhance the antinociceptive vs. rate-suppressant effects of mu-opioid agonists.

2. MATERIALS AND METHODS

2.1 Subjects

A total of seven middle-aged adult (10-18 years old) male rhesus macaques (*Macaca mulatta*) of either Indian or Chinese origin and weighing between 10-14 kg served as subjects. Four monkeys served as subjects in the assay of thermal nociception, and three monkeys served as subjects in the assay of schedule-controlled responding. These sample sizes have been sufficient to detect mu-opioid agonist interactions in previous publications (Banks et al., 2010a; Banks et al., 2010b; Maguire and France, 2014; Schwienteck et al., 2018; Stevenson et al., 2003). All monkeys had experimental histories of opioid, monoamine transporter ligand, and *N*-methyl *D*-aspartate antagonist exposure. Diet was comprised of laboratory monkey chow (#5049, Purina, Framingham, MA) and supplemented with fresh fruits, vegetables, and nuts. All subjects were housed individually and had *ad lib* water access while in the housing chamber. A 12h light/dark cycle (lights on from 6:00 AM to 6:00 PM) was in effect. Housing facilities were licensed by the United States Department of Agriculture and accredited by AAALAC International. The VCU Institutional Animal Care and Use Committee approved all research and enrichment protocols in accordance with the 2011 Guide for the Care and Use of Laboratory Animals.

2.2 Assay of Thermal Nociception

Monkeys were trained to sit comfortably in an acrylic restraint chair using the pole-and-collar technique such that their tails hung freely. The subject's tail was shaved 10-12 cm from the distal end weekly and immersed in a thermal container of warm water. If the subject did not

remove its tail by 20 s, the experimenter removed the tail and a latency of 20 s was assigned. A stopwatch was utilized to record tail-withdrawal latencies. During each 15-min cycle, tail-withdrawal latencies were recorded from water warmed to 38°C, 50°C, and 54°C and the order of warmed water presentations was counterbalanced between successive cycles. Baseline tail-withdrawal latencies at all three thermal intensities were determined in each daily test session before drug administration. Test sessions continued only if tail-withdrawal latencies from 38°C water did not occur before the 20 s cutoff. This criterion was met in every monkey during every test session. Time course test sessions consisted of a single drug dose administered intramuscularly (IM) and tail withdrawal latencies were re-determined at 10, 30, and 100 min post-drug administration. Cumulative dose test sessions consisted of four to five 15-minute cycles composed of a 10-minute drug pretreatment phase and a 5-min testing phase. Drugs were administered IM at the start of each 15-min cycle, and each drug dose increased the total cumulative dose by one-fourth or one-half log units. Tail-withdrawal latencies were re-determined during the 5-min testing phase as described above.

Initially, the time course of (−)-Ro 64-6198 (0.1 and 0.32 mg/kg) and SB-612111 (0.32 mg/kg) were singly determined. Following these initial Ro 64-6198 time-course experiments, two additional experiments were conducted. First, the effectiveness of Ro 64-6198 to alter the antinociceptive effects of 6 mu-opioid ligands and the kappa-opioid agonist nalfurafine was determined. Cumulative dose-effect functions for NAQ (0.1-3.2 mg/kg), buprenorphine (0.032-1 mg/kg), nalbuphine (0.032-3.2 mg/kg), morphine (0.1-10 mg/kg), oxycodone (0.01-1 mg/kg), methadone (0.1-5.6 mg/kg), and nalfurafine (0.0001-0.01 mg/kg) were determined following a 30-min pretreatment of 0.1 mg/kg Ro 64-6198 or vehicle. Mu-opioid ligands were tested up to doses that produced maximal antinociception, undesirable effects such as respiratory depression,

or reached solubility limits. Nalfurafine was tested up to doses that produced emesis. These experiments were generally conducted twice per week, except for studies with buprenorphine, nalbuphine, and nalfurafine, which were separated by at least 6 days to allow dissipation of long-acting drug effects and/or to minimize potential effects of antinociceptive tolerance. Ro 64-6198 test sessions were also separated by at least 7 days. Second, potency, time course, and antagonism of Ro 64-6198 enhancement of nalbuphine-induced antinociception were determined in three of the four monkeys used for the mu-opioid ligand and Ro 64-6198 interactions described above. One monkey was removed from this set of experiments due to health issues unrelated to the study. These experiments were also separated by at least 7 days. The experimenter was not blinded to drug or dose conditions due to potential animal health issues when evaluating novel drug interactions consistent with our previous publications (Banks et al., 2010a; Banks et al., 2010b; Cornelissen et al., 2018a). Ro 64-6198 and vehicle pretreatments were counterbalanced between opioid ligands, but pretreatments were consistent across all monkeys.

2.3 Assay of Schedule-Controlled Responding

Experiments were conducted in each monkey's housing chamber, which also served as the experimental chamber as previously described (Banks et al., 2010b). A custom-fabricated operant response panel and a food pellet dispenser (Med Associates, ENV-203-1000, St. Albans, VT) were attached to the front of the housing chamber. Panels were operated under a MED-PC interface and programmed with a Windows-based computer using MEDSTATE Notation (MED Associates). Training sessions were composed of five 15-min cycles for a total session duration of 75 min. Two components were incorporated into each cycle. The first component was a 10-

min time-out period during which responses had no scheduled consequences. The second component was a 5-min response period during which the right key was transilluminated red, and subjects could respond under a fixed-ratio 30 (FR30) schedule of food pellet presentation. If a subject earned the maximum of 10 pellets prior to completion of the 5-min period, the response component was terminated, stimulus lights were extinguished, and further responses resulted in no consequences. All monkeys were trained until rates of responding were ≥ 1.0 response/s during all 5 cycles for 7 consecutive days (data not shown).

Behavioral sessions were conducted 5 days per week. Training sessions were conducted on Mondays, Wednesdays, and Thursdays, and test sessions were conducted on Tuesdays and Fridays. Subjects were eligible for participation in test sessions if rates of operant responding were ≥ 1.0 response/s on training days that preceded test days. On test days, test compounds were administered IM using the same cumulative dosing procedure described above in the assay of thermal nociception. All drugs and pretreatment combinations were tested up to doses that either decreased responding >70% of the preceding training day's average response rate or reached solubility limits. Individual test sessions lasted for 3 to 6 cycles depending on individual subject behavior and treatment condition.

Initially, the potency and time course of vehicle, (–)-Ro 64-6198 (0.1-0.32 mg/kg), and SB- 612111 (0.32 mg/kg) were determined. Additionally, the effectiveness of SB-612111 to antagonize the rate-decreasing effects of Ro 64-6198 was evaluated. Subsequently, Ro 64-6198 interactions with the same opioid ligands evaluated in the assay of thermal nociception were determined. Cumulative dose-effect functions for NAQ (0.1-10 mg/kg), buprenorphine (0.032-3.2 mg/kg), nalbuphine (0.032-1 mg/kg), morphine (0.1-5.6 mg/kg), oxycodone (0.01-1.0 mg/kg), methadone (0.1-3.2 mg/kg), and nalfurafine (0.0001-0.0032 mg/kg), were determined

following a 30-min pretreatment of 0.1 mg/kg Ro 64-6198 or vehicle. These experiments were generally conducted twice per week, except for studies with buprenorphine, nalbuphine, and nalfurafine, which were separated by at least 7 days to allow dissipation of long-acting drug effects and/or to minimize potential tolerance to the rate-decreasing effects of low efficacy mu-opioid ligands. Ro 64-6198 test sessions were also separated by at least 7 days. Ro 64-6198 and vehicle pretreatments were counterbalanced between opioid ligands, but pretreatments were consistent across all monkeys.

2.4 Data Analysis

For the assay of thermal nociception, tail-withdrawal latencies (in sec) were converted to percent maximal possible effect (%MPE). %MPE was defined as {(Test latency – Control latency) ÷ (20 – Control Latency) * 100} where "test latency" was the latency in response to either 50°C or 54°C at each dose during the cumulative dosing procedure, and "control latency" was the latency in response to either 50°C or 54°C taken during the baseline period prior to drug administration. Statistical analysis of all %MPE data was conducted using a repeated-measures two-way ANOVA with either time or pretreatment and opioid ligand dose as the main factors (all factors repeated measures). A Sidak or Tukey post-hoc test, as appropriate, followed all significant interactions. Significance was set *a priori* at the 95% confidence level.

For the assay of schedule-controlled responding, rates of operant responding (responses/sec) during each test cycle were converted to percent control rate using the average rate of responding of the 5 cycles from the individual monkey's previous training session. Statistical analysis of all % control data was conducted using a repeated-measures two-way ANOVA with either time or pretreatment and opioid ligand dose as the main factors (all factors

repeated measures). A Sidak or Tukey post-hoc test, as appropriate, followed all significant interactions.

In addition, the effective dose (ED_{50}) that produced either 50%MPE or 50% reduction in control rates of responding was determined for each mu-opioid ligand and nalfurafine following 0.1 mg/kg Ro 64-6198 or vehicle pretreatment. ED50 values were determined by interpolation when only 2 data points were available (one below and one >50% effect) or by linear regression when at least 3 data points on the linear portion of the dose-effect function were available as previously described (Banks et al., 2010b; Cornelissen et al., 2018a; Cornelissen et al., 2018b; Stevenson et al., 2003). Individual ED_{50} values were subsequently averaged to yield group mean ED_{50} values and 95% confidence intervals using the Student's T distribution (confidence.t equation in Microsoft Excel for Mac, Version 16.9, Microsoft, Redmond, WA).

2.5 Drugs

(±)-Methadone HCl and (±)-buprenorphine HCl were purchased from a commercial supplier (Spectrum Chemicals, Gardena, CA). (−)-Oxycodone HCl, (−)-morphine sulfate, (−)-nalfurafine HCl, (−)-Ro 64-6198 HCl, and SB-612111 HCl were supplied by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). (−)-Nalbuphine HCl was supplied by Dr. Kenner Rice (Drug Design and Synthesis Section, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). NAQ HCl was synthesized as previously described (Li et al., 2009) and supplied by Drs. Samuel Obeng and Yan Zhang. Buprenorphine, nalbuphine, oxycodone, morphine, methadone, and nalfurafine were dissolved in sterile water. (−)-Ro 64-6198 was dissolved in a solution of 1:4:5 Tween 80 (Spectrum Chemicals, Gardena, CA) to dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis,

MO) to sterile water. SB-612111 was dissolved in a solution of 4:6 DMSO to sterile water. NAQ was dissolved in a solution of 3:7 DMSO to sterile water. All drug doses were administered intramuscularly and expressed as the salt forms listed above.

3. RESULTS

3.1 Effects of Ro 64-6198 alone

Average ± S.E.M. baseline tail withdrawal latencies for Ro 64-6198 alone and in combination with various mu-opioid ligand experiments were 1.0 ± 0.4 s at 50°C and 0.7 ± 0.1 s at 54°C. Average control rates of responding across all experiments was 2.5 ± 0.4 responses/s. Fig. 1 shows the potency and time course of Ro 64-6198 alone to produce antinociception (panels A and B) and rate-suppression (panel C). Up to 0.32 mg/kg, Ro 64-6198 did not produce significant antinociception with a maximum %MPE of 9.4 ± 7.4 and 1.1 ± 0.8 at 50 and 54°C, respectively. In contrast, Ro 64-6198 produced dose- and time-dependent decreases in rates of responding with maximal effects at 100 min following 0.32 mg/kg administration (treatment: $F_{4,8}$=16.2, P<0.05; time: $F_{3,6}$=18.8, P<0.05; interaction: $F_{12,24}$=2.9, P<0.05). The rate-decreasing effects of Ro 64-6198 were blocked by the NOP antagonist SB-612111 at a dose (0.32 mg/kg) that had no effect on rates of responding alone. Larger Ro 64-6198 doses (1.0 mg/kg) were evaluated, but required prompt SB-612111 administration due to the emergence of undesirable effects including loss of muscle tone and slowed respiratory rate.

3.2 Effects of Ro 64-6198 pretreatment on mu-opioid ligand-induced antinociception

Fig. 2 shows the antinociceptive effects of NAQ, buprenorphine, nalbuphine, morphine, oxycodone, and methadone following either vehicle or 0.1 mg/kg Ro 64-6198 pretreatment.

Buprenorphine, nalbuphine, morphine, oxycodone, and methadone produced dose-dependent antinociception at 50°C under vehicle conditions and the corresponding ED_{50} values for each mu-opioid agonist are reported in Table 1. NAQ produced a group mean ± S.E.M. maximum %MPE of 4.9±5.1 at 50°C (Fig. 2, panel A). Supplemental Fig. 1 also shows the antinociceptive effects of these same mu-opioid ligands alone at a higher thermal intensity (54°C). Only morphine, oxycodone, and methadone produced > 50%MPE at 54°C and the corresponding ED_{50} values are reported in Supplemental Table 1. NAQ, buprenorphine, and nalbuphine produced a maximum %MPE of 1.1±1.9, 9.3±6.9, and 37.2±7.3 at 54°C, respectively (Supplemental Table 1).

Pretreatment with a Ro 64-6198 dose (0.1 mg/kg) that was ineffective alone significantly enhanced the antinociceptive effects of buprenorphine (dose: $F_{2,6}$= 29.4, P<0.05; pretreatment: $F_{1,3}$=17.4, P<0.05; interaction: $F_{2,6}$=17.0, P<0.05), nalbuphine (dose: $F_{4,12}$= 489, P<0.05; pretreatment: $F_{1,3}$=79.3, P<0.05; interaction: $F_{4,12}$=19.8, P<0.05), and methadone (dose: $F_{4,12}$=56.6, P<0.05; interaction: $F_{4,12}$=4.2, P<0.05) at 50°C (Fig. 2). The corresponding ED_{50} values of each mu-opioid agonist following Ro 64-6198 pretreatment are also reported in Table 1. Post-hoc power analyses indicated the morphine (power=0.65) and oxycodone (power=0.67) experiments were underpowered to detect a significant interaction between Ro 64-6198 and these two mu-opioid agonists. At the 54°C thermal stimulus, Ro 64-6198 pretreatment also significantly enhanced the antinociceptive effects of methadone (methadone dose: $F_{4,12}$=657, P<0.05; pretreatment: $F_{1,3}$=146.4, P<0.05; interaction: $F_{4,12}$= 65.1, P<0.05). The corresponding ED_{50} values for methadone and the other mu-opioid agonists at 54°C are also reported in Supplemental Table 1.

3.3 Effects of Ro 64-6198 pretreatment on mu-opioid ligand-induced rate-suppression

Fig. 3 shows the effects of NAQ, buprenorphine, nalbuphine, morphine, oxycodone, and methadone on rates of responding following either vehicle or 0.1 mg/kg Ro 64-6198 pretreatment. NAQ and buprenorphine alone did not significantly alter rates of responding up to the largest doses tested and maximal rate-decreasing effects (mean ± S.E.M.) were 87.4 ± 27.9% and 64.9 ± 20.8% control, respectively (Fig. 3). Larger NAQ and buprenorphine doses could not be examined due to solubility limits. However, NAQ and buprenorphine were tested up to doses that antagonized the antinociceptive effects of other mu-opioid agonists (Cornelissen et al., 2018a; Walker et al., 1995). Nalbuphine, morphine, oxycodone, and methadone alone all produced dose-dependent decreases in rates of responding and the corresponding ED_{50} values are shown in Table 1. Ro 64-6198 pretreatment did not enhance the effectiveness of NAQ or buprenorphine to alter rates of responding (Fig. 3). Ro 64-6198 pretreatment also did not enhance the potency of nalbuphine, morphine, oxycodone, or methadone to decrease rates of responding as denoted by overlapping confidence limits for ED_{50} values (Table 1).

3.4 Effects of Ro 64-6198 pretreatment on nalfurafine-induced antinociception and rate-suppression

Fig. 4 shows the antinociceptive (A) and rate-altering (B) effects of nalfurafine following either vehicle or 0.1 mg/kg Ro 64-6198 pretreatment. Nalfurafine alone failed to produce antinociception up to the highest dose tested with maximal effects (mean ± S.E.M.) of 9.1 ± 10.7 % and 6.8 ± 2.9 % at 50 and 54°C, respectively (Fig. 4 panels A and B). Ro 64-6198 enhanced the antinociceptive effects of nalfurafine at 50°C (nalfurafine dose: $F_{4,8}$=4.7, P<0.05; interaction: $F_{4,8}$= 4.8, P<0.05) (Fig. 4 panel A). Nalfurafine alone produced dose- and time- dependent

decreases in rates of responding and the corresponding ED_{50} values are shown in Table 1. Time course of nalfurafine rate-decreasing effects are shown in Supplemental Fig. 2. Ro 64-6198 pretreatment attenuated the effectiveness of cumulative 0.001 mg/kg nalfurafine to decrease rates of responding (nalfurafine dose: $F_{3,6}$=11.7, P<0.05; interaction: $F_{3,6}$= 8.9, P<0.05). Ro 64-6198 did not alter nalfurafine potency to decrease rates of responding (Table 1).

3.5 Potency, time course, and antagonism of Ro 64-6198 enhancement of nalbuphine antinociception

Fig. 5 shows the potency (A), time course (B), and sensitivity to NOP antagonism (C) of Ro 64-6198 enhancement of nalbuphine antinociception at 50°C. For these experiments, group mean ± S.E.M. baseline tail withdrawal latencies were 0.8 ± 0.3 s at 50°C and 0.8 ± 0.2 s at 54°C. There was no significant effect of Ro 64-6198 on nalbuphine effects at 54°C (data not shown). Similar to results in Fig. 2, 0.1 mg/kg Ro 64-6198, but not 0.032 mg/kg, enhanced nalbuphine antinociception (nalbuphine dose: $F_{4,8}$=39.3, P<0.05; Ro 64-6198 dose: $F_{2,4}$= 12.1, P<0.05; interaction: $F_{8,16}$= 8.3, P<0.05) (Fig. 5 panel A). In addition, only the 30-min pretreatment time was sufficient for Ro 64-6198 to enhance the antinociceptive effects of nalbuphine (time: $F_{3,6}$=6.3, P<0.05; nalbuphine dose: $F_{4,8}$=61.3, P<0.05; interaction: $F_{12,24}$=6.8, P<0.05) (Fig. 5 panel B). Finally, Ro 64-6198 enhancement of nalbuphine antinociception was blocked by the NOP antagonist SB-612111 (nalbuphine dose: $F_{4,8}$=53.5, P<0.05; pretreatment: $F_{3,6}$=11.7, P=0.05; interaction: $F_{12,24}$=9.3, P<0.05) (Fig. 5 panel C). SB-612111 (0.32 mg/kg) pretreatment also significantly attenuated the antinociceptive effects of the 0.32 mg/kg cumulative nalbuphine dose (Fig. 5 panel C).

4. DISCUSSION

4.1 Conclusions

The present study determined whether mu-opioid ligand efficacy was a determinant of antinociceptive interactions with the NOP agonist (−)-Ro 64-6198 in rhesus monkeys. There were three main findings. First, both Ro 64-6198 and nalfurafine were more potent to decrease rates of responding than produce antinociception. Second, Ro 64-6198 enhanced the antinociceptive potency of buprenorphine, nalbuphine, and methadone suggesting that mu-opioid agonist efficacy was not a determinant of mu-opioid and NOP agonist interactions. Despite, Ro 64-6198 and mu-opioid agonist interactions displaying some degree of behavioral selectivity, Ro 64-6198 enhancement of mu-agonist antinociception occurred under a narrow range of experimental conditions. Lastly, NOP agonist interactions were not selective for mu-opioid agonists because Ro 64-6198 also enhanced the antinociceptive effects of the kappa-opioid agonist nalfurafine. Collectively, these results dampen enthusiasm for NOP agonists as candidate "opioid-sparing" adjuncts.

4.2 Effects of Ro 64-6198, mu-opioid agonists, and nalfurafine alone

The mu-opioid agonists buprenorphine, nalbuphine, morphine, oxycodone, and methadone produced dose- and noxious stimulus- dependent antinociception consistent with the extant literature (Cornelissen et al., 2018a; Gatch et al., 1998; Maguire and France, 2014; Walker et al., 1993). Nalfurafine failed to produce antinociception up to a 3-fold larger dose than doses that suppressed rates of responding. Although nalfurafine has been previously shown to produce antinociception in a warm water tail-withdrawal procedure in monkeys (Endoh et al., 2001; Ko and Naughton, 2009), nalfurafine-induced antinociception in these previous studies occurred at

doses larger than those that maximally decreased rates of responding in the present study. The NOP agonist Ro 64-6198 also did not produce antinociception up to doses that significantly decreased rates of responding. These results were consistent with a previous monkey study (Saccone et al., 2016), but inconsistent with other monkey studies (Cremeans et al., 2012; Ko et al., 2009; Podlesnik et al., 2011). Reasons for the inconsistent NOP agonist antinociceptive effects in monkeys are not entirely clear and highlight the importance of evaluating candidate analgesics across a broad range of experimental conditions. One potential explanation for the differential Ro 64-6198 antinociceptive results could be related to the experimental and pharmacological histories of the monkeys. For example, monkeys in the Ko, et al (2009) study had not been exposed to any opioid ligands for at least one month prior whereas monkeys in the present study had a more extensive and recent opioid ligand history (Cornelissen et al., 2018a; Cornelissen et al., 2018b). Thus, one interpretation could be that NOP agonists produce antinociception in opioid-naïve or minimally opioid-experienced primates. Although opioid ligand history did not impact the antinociceptive effects of mu-opioid ligands alone in the present study, the degree to which opioid ligand exposure may alter the antinociceptive effects of NOP agonists remains to be empirically determined.

The opioid agonists nalbuphine, morphine, oxycodone, and methadone decreased rates of responding consistent with the extant literature (Banks et al., 2010b; Downs, 1979; Stevenson et al., 2003). The present results extend these findings to the KOR agonist nalfurafine. NAQ failed to significantly alter rates of responding in the present study. Previous studies have shown that NAQ decreases rates of food-maintained responding (Siemian et al., 2016) and to a lesser extent, electrical brain stimulation-maintained responding (Altarifi et al., 2015) in rats suggesting potential species difference in NAQ effectiveness to decrease operant behavior. Buprenorphine

also failed to significantly alter rates of responding and these results were consistent with previous buprenorphine results in male monkeys (Negus et al., 2002). Ro 64-6198 rate-decreasing effects in the present study were consistent with previous Ro 64-6198 results in drug discrimination (Saccone et al., 2016) and extended previous findings by determining Ro 64-6198 time course and sensitivity to SB-612111antagonism. Overall, the behavioral effects of the mu-opioid ligands and nalfurafine alone in the present study provide an empirical foundation for examining interactions with Ro 64-6198.

4.3 Interactions between Ro 64-6198 and mu-opioid or kappa-opioid agonists

Ro 64-6198 significantly enhanced the antinociceptive effects of the mu-opioid ligands buprenorphine, nalbuphine, and methadone as well as nalfurafine. The present results were generally consistent with the direction, but not the magnitude, of previous mu-opioid and NOP agonist antinociceptive interactions with buprenorphine (Cremeans et al., 2012) and morphine (Hu et al., 2010; Ko and Naughton, 2009) in monkeys. NOP agonist enhancement of morphine antinociception has also been reported in mice (Reiss et al., 2008) and rats (Jin-Hua et al., 1997). The present results extended upon these previous findings in three ways. First, NOP and mu-opioid agonist antinociceptive interactions were not dependent upon mu-opioid agonist efficacy. Second, mu-opioid and NOP agonist antinociceptive interactions occurred under a narrow range of experimental conditions such as dose and pretreatment time that suggests limited clinical utility and effectiveness. Lastly, NOP agonist interactions were not selective for clinically effective mu-opioid agonists because Ro 64-6198 also enhanced nalfurafine-induced antinociception.

In contrast to mu-opioid and NOP agonist antinociceptive interactions, Ro 64-6198 did

not significantly alter the rate-decreasing effects of any mu-opioid ligand examined. However, Ro 64-6198 significantly attenuated the rate-decreasing effects of cumulative 0.001mg/kg nalfurafine. These results suggest at least three main conclusions. First, Ro 64-6198 enhancement of mu-opioid agonist antinociception was not due to generalized behavioral depression. However, one caveat is the Ro 64-6198 dose sufficient to enhance mu-opioid agonist antinociception was only 3-fold smaller than the dose that significantly decreased rates of responding. Thus, there may be a potential ceiling for the amount of NOP agonist in the NOP/mu-opioid drug mixture. Second, the present results are consistent with and extend previous NOP and mu-opioid agonist interactions to the mu-opioid agonist undesirable endpoint behavioral depression. Previous studies have reported that NOP agonists do not enhance the respiratory depressant, scratching-behavior, or reinforcing effects of mu-opioid agonists in monkeys (Cremeans et al., 2012; Ko et al., 2009; Podlesnik et al., 2011). Third, although Ro 64-6198 attenuated the rate-decreasing effects of cumulative 0.001 mg/kg nalfurafine, nalfurafine produced maximal rate-depression at similar doses irrespective of pretreatment. Overall, despite mu-opioid and NOP agonist interactions displaying some degree of behavioral selectivity to produce antinociception vs. rate suppression, the magnitude of these interactions were small and not systematic across the various mu-opioid agonists.

4.4 Comparison to mu-opioid Agonist and Other Drug Interactions

Similar to mu-opioid and NOP agonist interactions, cannabinoid receptor agonists, delta-opioid agonists, serotonin uptake inhibitors and serotonin receptor agonists have also produced a selective enhancement of mu-opioid agonist antinociception in rhesus monkeys (Banks et al., 2010b; Gatch et al., 1998; Maguire and France, 2014; Negus et al., 2009; Stevenson et al., 2003).

For example, the serotonin uptake inhibitor clomipramine enhanced the antinociceptive effects of low efficacy mu-opioid agonists to a greater extent than high efficacy mu-opioid agonists (Banks et al., 2010b; Gatch et al., 1998). In contrast, cannabinoid agonists enhanced the antinociceptive effects of high efficacy mu-opioid agonists to a greater degree than low efficacy mu-opioid agonists (Maguire and France, 2014). Furthermore, delta agonist enhancement of mu-opioid agonist antinociception did not depend on mu-opioid agonist efficacy similar to the present NOP agonist effects (Negus et al., 2009; Stevenson et al., 2003). Overall, this literature supports 1) the inclusion of multiple dependent measures to assess behavioral selectivity in preclinical analgesia drug development and 2) the systematic evaluation of behavioral interactions with mu-opioid ligands that vary in efficacy.

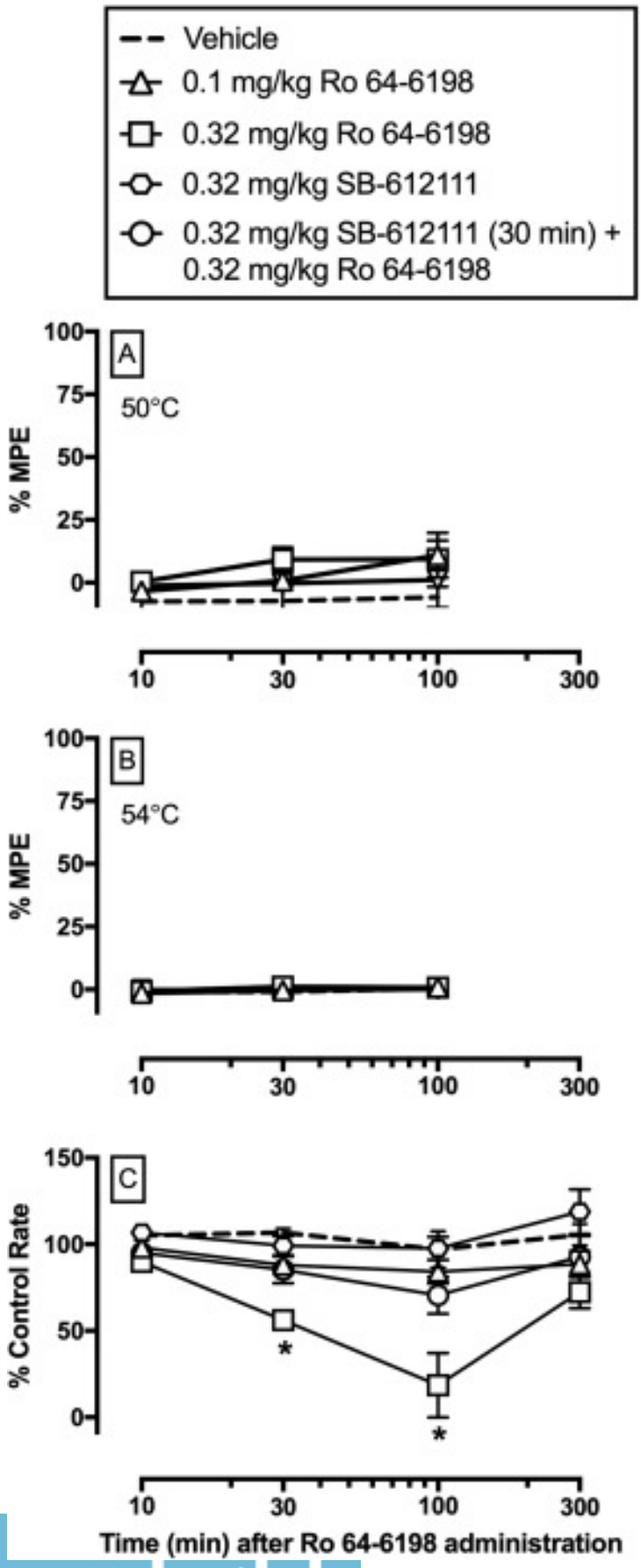


Fig 1

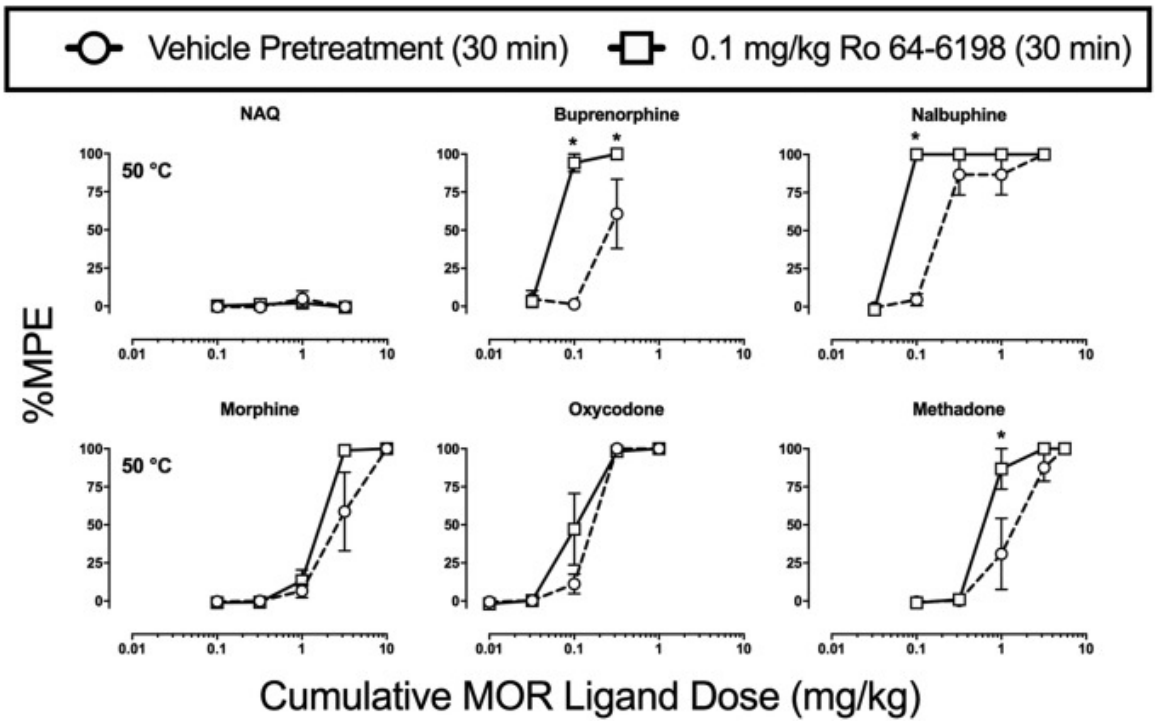


Figure 2

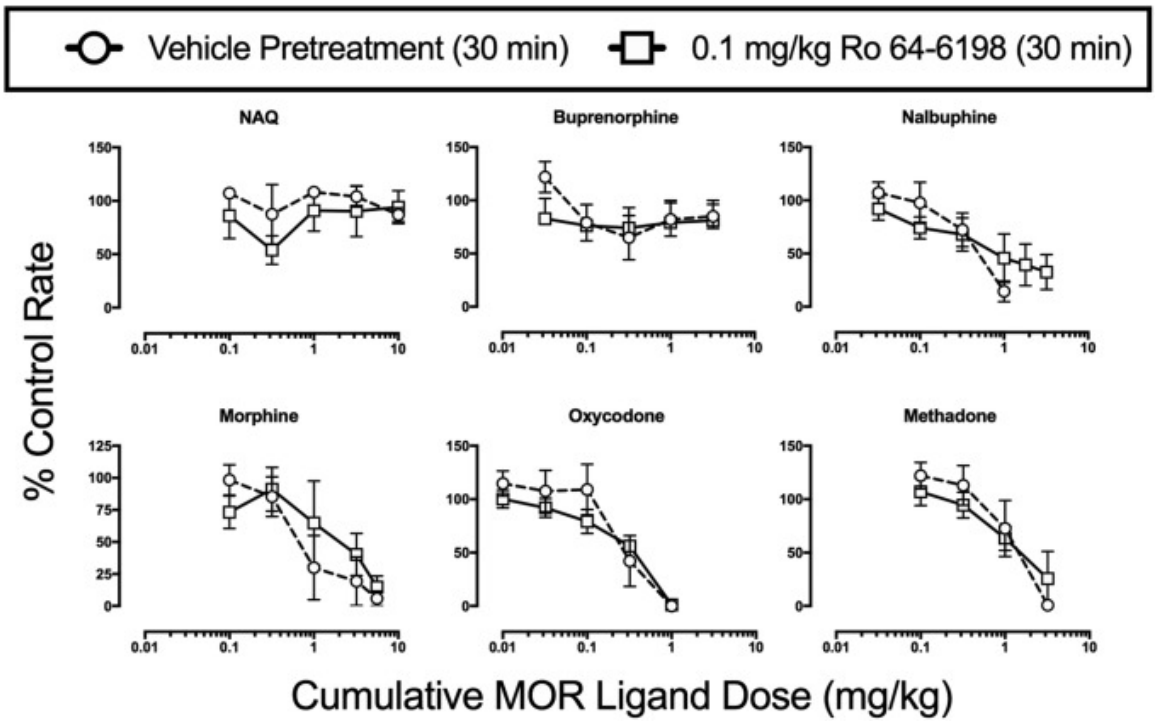


Fig 3

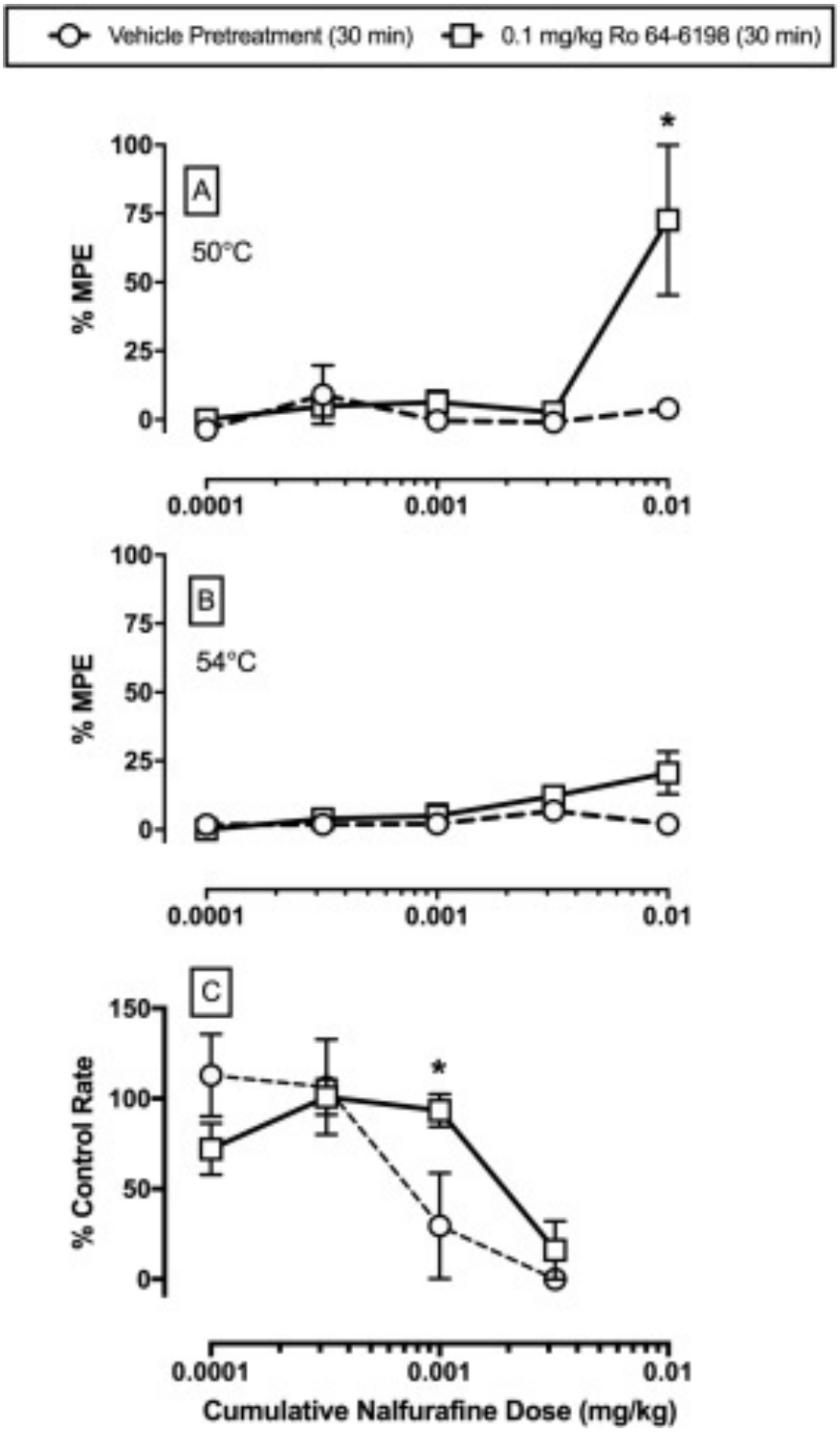


Fig 4

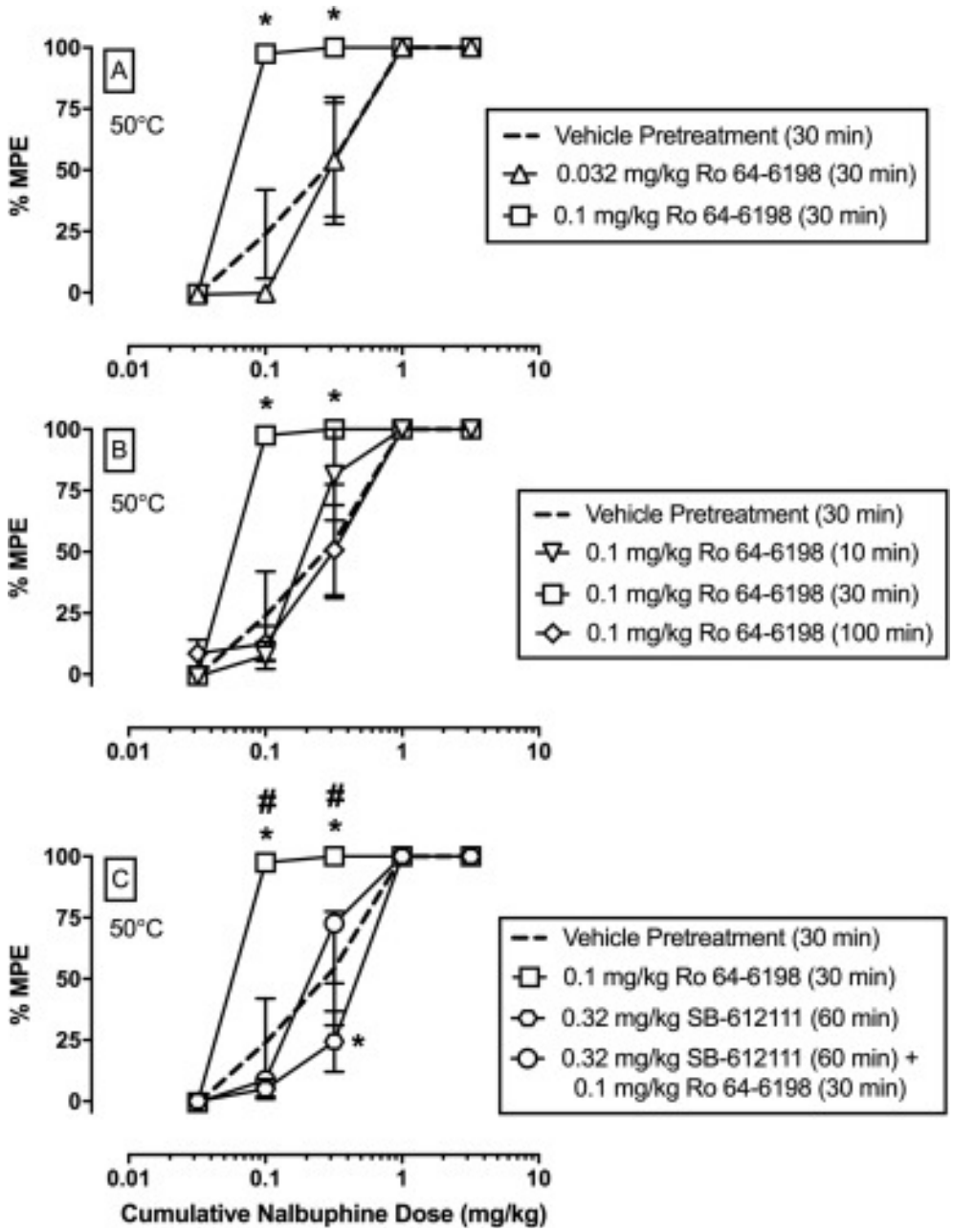


Fig 5

Legends for Figures

Fig 1: Time course of (−)-Ro 64-6198 and SB-612111 in assays of warm-water tail withdrawal at 50°C (panel A) and 54°C (panel B) and schedule-controlled responding (panel C). Abscissae: time in min after intramuscular Ro 64-6198 administration (log scale). Ordinates: percent maximal possible effect (panels A and B) or percent control rate (panel C). All points represent mean ± S.E.M. of 4 monkeys (except panel C where n=3). Asterisks denote significant ($P<0.05$) difference from vehicle and 0.32 mg/kg (−)-Ro 64-6198 + 0.32 mg/kg SB-612111.

Fig 2: Antinociceptive effects (50°C) of NAQ, buprenorphine, nalbuphine, morphine, oxycodone, and methadone following vehicle or 0.1 mg/kg (−)-Ro 64-6198 pretreatment. Abscissae: cumulative intramuscular mu-opioid ligand dose in milligrams per kilogram (log scale). Ordinates: percent maximal possible effect (%MPE). All points represent mean ± S.E.M. of 4 monkeys (except NAQ and morphine which is n=3). Asterisks denote significant ($P<0.05$) difference from vehicle.

Fig 3: Rate-decreasing effects of NAQ, buprenorphine, nalbuphine, morphine, oxycodone, and methadone following vehicle or 0.1 mg/kg (−)-Ro 64-6198 pretreatment. Abscissae: cumulative intramuscular mu-opioid ligand dose in milligrams per kilogram (log scale). Ordinates: percent control rate. All points represent mean ± S.E.M. of 3 monkeys. Asterisks points denote significant ($P<0.05$) difference from vehicle.

Fig 4: Antinociceptive (50°C; Panel A) and rate-decreasing (Panel B) effects of nalfurafine following vehicle or 0.1 mg/kg (−)-Ro 64-6198 pretreatment. Abscissae: cumulative

intramuscular nalfurafine dose in milligrams per kilogram (log scale). Ordinates: percent maximal possible effect (%MPE; panel A) or percent control rate of responding (panel B). Points in panel A represent mean ± S.E.M. of 4 monkeys and panel B represent mean ± S.E.M. of 3 monkeys. Asterisks denote significant ($P<0.05$) difference from vehicle.

Fig 5: Antinociceptive effects (50°C) of nalbuphine following vehicle, (−)-Ro 64-6198, or SB 612111 pretreatment. Panel A shows effects of different Ro 64-6198 doses. Panel B shows effects of different Ro 64-6198 pretreatment times. Panel C shows sensitivity to the NOP antagonist SB-612111. Abscissae: cumulative intramuscular nalbuphine dose in milligrams per kilogram (log scale). Ordinates: percent maximal possible effect (%MPE). All points represent mean ± S.E.M. of 3 monkeys. Asterisks denote significant ($P<0.05$) difference from vehicle. # denote significant difference ($P<0.05$) from Ro 64-6198 + SB-612111.

Table 1: Opioid ligand ED_{50} values (95% confidence limits; CL) following vehicle or 0.1 mg/kg (–)-Ro 64-6198 pretreatment in assays of thermal nociception (TW) and schedule-controlled responding (SCR) in male rhesus monkeys. All values represent group mean ED_{50} values of 3 (SCR) or 4 (TW) monkeys unless otherwise denoted. [a] ED_{50} values are from three out of four monkeys.

Opioid ligand	**TW (50°C)** **ED_{50} in mg/kg (95% CL)**	**SCR** **ED_{50} in mg/kg (95% CL)**
Methadone	1.45 (1.01-1.89)	1.17 (0.66-1.68)
+ Ro 64-6198	0.79 (0.52-1.06)	1.19 (0.63-1.74)
Oxycodone	0.16 (0.02-0.30)	0.24 (0-1.14)
+ Ro 64-6198	0.10 (0-0.45)	0.29 (0.13-0.45)
Morphine (n=3)	3.27 (0-7.76)	0.74 (0-1.89)
+ Ro 64-6198 (n=3)	1.55 (0.86-2.25)	2.17 (0-6.10)
Nalbuphine	0.20 (0.04-0.36)	0.42 (0-1.19)
+ Ro 64-6198	0.05 (0.05-0.07)	0.83 (0-2.88)
Buprenorphine	0.28 (0-0.62)[a]	NC
+ Ro 64-6198	0.06 (0-0.30)	NC
NAQ	NC	NC
+ Ro 64-6198	NC	NC
Nalfurafine (n=3)	NC	0.001 (0.001-0.002)
+ Ro 64-6198 (n=3)	0.006 (0-0.012)	0.002 (0.001-0.014)

NC: not calculable because no drug dose produced >50%MPE or decreased %Control rate below 50%

Chapter 4: Expansion of the Furchgott equation for receptor theory by investigating the potential for fixed-proportion mixtures of competitive MOR antagonist and MOR agonist to manipulate antinociceptive efficacy in male rhesus monkeys.

INTRODUCTION

Pharmacodynamics is concerned with the affinity and efficacy of drugs at their receptor targets. Drug affinity can be precisely measured with ligand binding techniques, but drug efficacy to activate receptor signaling and produce downstream effects is a relative measure dependent in part on the signaling pathway(s) and downstream effects under consideration, and drug efficacies are typically described in relation to some standard high-efficacy ligand (Ruffolo, 1982; Kenakin, 2012). Although efficacy is challenging to measure, it is clearly relevant in drug development. For example, mu-opioid receptor (MOR) ligands differ in their efficacy to activate MOR-coupled signal transduction processes and produce MOR-mediated effects such as analgesia and respiratory depression. Fentanyl has high MOR efficacy, and increasing fentanyl doses can produce both antinociception and lethal respiratory depression (Gerak et al., 1994; Banks et al., 2010a; Ding et al., 2016). At the other extreme of the efficacy continuum, naltrexone has little or no MOR efficacy, produces no agonist effects, and functions as a competitive reversible antagonist (Walker et al., 1994; Ko et al., 1998; Bowen et al., 2002). Between these extremes are intermediate-efficacy MOR ligands like nalbuphine and buprenorphine that produce submaximal stimulation of MOR signaling and a subset of agonist effects that includes analgesia but only weak respiratory depression (Gerak et al., 1994; Pitts et al., 1998; Kishioka et al., 2000). Experiments to investigate the expression and consequences of ligand efficacy at MORs or other receptor targets can be useful both (a) to determine the efficacy

required to produce different effects of interest, and (b) to evaluate relative efficacy of new ligands as they are developed.

One common approach to efficacy evaluations relies on the use of irreversible antagonists to evaluate the impact of reducing receptor number on expression of drug effects (Furchgott, 1966; Kenakin, 1993; Bergman et al., 2000). Efficacy requirements for different effects can be estimated, because an irreversible antagonist will produce greater antagonism of effects with high vs. low efficacy requirements (Zernig et al., 1997). Relative efficacies of different drugs can be estimated, because an irreversible antagonist will produce greater antagonism of a low- vs. high-efficacy agonist (Zimmerman et al., 1987; Walker et al., 1998). However, studies with irreversible antagonists can be logistically challenging (e.g. due to the long duration of antagonist effects), and irreversible antagonists are not available for many receptors of interest. Decreases in receptor number can also be accomplished with genetic mutations, as in wild-type, heterozygous, and homozygous receptor knockout animals (Grim et al., 2016), but the degree of control over the magnitude of that decrease is limited. Receptor theory suggests an alternative, more precise, and more flexible strategy to investigate efficacy using mixtures of competitive agonists and antagonists. Figure 1A shows a theoretical dose-effect function for a high-efficacy agonist administered alone or in the presence of increasing fixed doses of an antagonist. The familiar result is an antagonist dose-dependent rightward shift in the agonist dose-effect curve (Ko et al., 1998; Negus et al., 2003). Figure 1B shows theoretical effects using a different experimental design, in which the agonist is administered in combination with fixed-proportional doses of the antagonist, such that increasing agonist doses are administered in combination with increasing antagonist doses. In this design, the antagonist is expected to produce proportion-dependent downward shifts in the agonist dose-effect curve, and mixtures with decreasing

agonist-to-antagonist proportions have decreasing apparent efficacies to activate the receptor. This approach has two potential advantages relative to existing strategies. First, agonist-to-antagonist proportion can be precisely manipulated to yield precise increments in efficacy. Second, this approach could be applied to any receptor system for which a competitive agonist and antagonist are available.

The goal of the present study was to test the utility of this approach using the competitive MOR agonist fentanyl and antagonist naltrexone (Negus et al., 1993; Emmerson et al., 1994; Walker et al., 1994; Emmerson et al., 1996). Effects of these drugs administered alone and in fixed-proportion mixtures were determined in an assay of thermal nociception using two thermal stimulus intensities (50 and 54°C warm water) and compared to effects produced by six other MOR ligands shown previously to vary in their relative MOR efficacies in *in vitro* assays of agonist-stimulated GTPγS binding (Emmerson et al., 1996; Selley et al., 1998; Alt et al., 2001; Thompson et al., 2004; Yuan et al., 2015). We predicted that effects of fentanyl, naltrexone, and the mixtures would match predicted results in Figure 1B. Additionally, we predicted that maximal effects of fentanyl, naltrexone, and the mixtures could be used to generate efficacy-effect scales for quantification of both (a) MOR efficacy requirements for antinociception at 50 and 54°C, and (b) relative efficacies of the six MOR test ligands.

METHODS

Subjects

Four adult male rhesus macaques (*Macaca mulatta*) of either Indian or Chinese origin and weighing between 10-14 kg served as subjects. All subjects had previous experimental histories that included exposure to opioid ligands, monoaminergic transporter ligands, and *N*-

methyl *D*-aspartate antagonists. Monkeys were fed a diet of laboratory biscuits (#5049, Purina, Framingham, MA) supplemented with fresh fruits, vegetables, and nuts to maintain healthy, stable body weights. Monkeys were individually housed in a temperature and humidity controlled room that was maintained on a 12-h light/12-h dark cycle (lights on from 6:00 AM until 6:00 PM). Water was available *ad libitum* in the housing chamber. The facility was licensed by the United States Department of Agriculture and accredited by AAALAC International. Both research and enrichment protocols were approved by the Institutional Animal Care and Use Committee and in accordance with the 2011 Guide for the Care and Use of Laboratory Animals. Environmental enrichment included: music, movies, puzzle feeders, and chew toys. Furthermore, monkeys were afforded opportunities to interact socially using olfactory and auditory cues; mirrors provided additional opportunities for visual interaction.

Assay of thermal nociception

Monkeys were trained to sit comfortably in an acrylic restraint chair using the pole-and-collar technique such that their tails hung freely. The subject's tail was shaved 10-12 cm from the distal end weekly and immersed in a thermal container of warm water. If the subject did not remove its tail by 20 s, the tail was removed by the experimenter, and a latency of 20 s was assigned. A stopwatch was utilized to record tail-withdrawal latencies. During each 15-min cycle, tail-withdrawal latencies were recorded from water warmed to 38°C, 50°C, and 54°C and the order of warmed water presentations varied between successive cycles. Baseline tail-withdrawal latencies at all three thermal intensities were determined in each daily test session before drug administration. Test sessions continued only if tail-withdrawal latencies from 38°C water did not occur before the 20-s cutoff. This criterion was met in every monkey during every

test session. Cumulative dose test sessions consisted of four to six 15-minute cycles composed of a 10-minute drug pretreatment phase and a 5-min testing phase. Drugs were administered intramuscularly (IM) at the start of each 15-min cycle, and each drug dose increase the total cumulative dose by one-fourth or one-half log units. Tail-withdrawal latencies were redetermined during the 5-min testing phase as described above.

Initially, dose-effect functions were determined for fentanyl (0.001-0.056 mg/kg, IM) and naltrexone (0.032-1 mg/kg, IM) alone and each dose-effect function was determined twice. Subsequently, three fixed-proportion fentanyl and naltrexone mixtures were examined and each cumulative dose-effect function was determined once. The proportions of each drug in the three test mixtures were based on the published affinities (K_d) of fentanyl (1.48 nM) and naltrexone (0.11 nM) at the mu-opioid receptor in rhesus monkey brain (Emmerson et al., 1994). Specifically, the fixed-proportion of fentanyl to naltrexone for one mixture, denoted as the 1:1 mixture, was set to the proportion of their K_d values (1.48:0.11 = 1:0.074). Relative to the 1:1 mixture, the 3:1 fentanyl/naltrexone mixture had a three-fold higher proportion of fentanyl to naltrexone (1:0.025), and the 1:3 fentanyl/naltrexone mixture had a three-fold lower proportion of fentanyl to naltrexone (1:0.22). Mixtures were tested up to doses that produced maximal antinociception, undesirable physiological effects such as respiratory depression, or antagonized fentanyl effects in other studies. Experiments were generally conducted twice per week, usually on Tuesdays and Fridays, with at least three days between test days.

Following these initial fentanyl/naltrexone fixed-proportion experiments, three additional studies were conducted. First, for comparison to effects of the fentanyl/naltrexone mixtures, cumulative dose-effect functions were determined for a series of six other MOR ligands that vary from low to high in their efficacy at mu receptors as determined by in vitro assays of agonist-

stimuluated GTPγS binding (Emmerson et al., 1996; Selley et al., 1998; Alt et al., 2001; Thompson et al., 2004; Yuan et al., 2015): 17-cyclopropylmethyl-3,14β-dihyroxy-4,5α-epoxy-6α-[(3′-isoquinolyl)acetamindo]morphinan (NAQ) (0.1-10 mg/kg, IM), buprenorphine (0.032-3.2 mg/kg, IM), nalbuphine (0.032-3.2 mg/kg, IM), morphine (0.1-10 mg/kg, IM), oxycodone (0.01-1 mg/kg, IM), and methadone (0.1-5.6 mg/kg, IM). Each dose-effect function was determined once. Drugs were tested up to doses that produced maximal antinociception, undesirable physiological effects such as respiratory depression, or antagonized fentanyl effects in other studies. These experiments were generally conducted twice per week, except for studies with buprenorphine, nalbuphine, and morphine, which were separated by at least 7 days to allow dissipation of long-acting drug effects and/or to minimize potential effects of antinociceptive tolerance. Second, receptor theory predicts that pretreatment with a low-efficacy agonist should attenuate the potency, but not efficacy, of a higher efficacy agonist and thus shift the higher efficacy agonist dose-effect function to the right. To test this hypothesis, fixed-dose pretreatment experiments were conducted with naltrexone (0.0032-0.032 mg/kg, IM), NAQ (10 mg/kg, IM), or 1:0.22 fentanyl/naltrexone mixture (0.032 mg/kg fentanyl + 0.007 mg/kg naltrexone, IM) to cumulative fentanyl (0.001-1 mg/kg, IM), and each experiment was singly determined. Naltrexone, NAQ, and the fentanyl/naltrexone mixture were administered 15 min before the first fentanyl dose. Lastly, drug interactions can be influenced not only by the relative drug doses in a mixture, but also by their relative time courses. Accordingly, the time course of 0.056 mg/kg fentanyl was determined when combined with naltrexone as a 1:0.074 fixed-proportion mixture for simultaneous administration of both drugs, and when the equivalent naltrexone dose (0.0041 mg/kg) in the 1:0.074 fentanyl/naltrexone mixture was administered 3 min before or 3 min after 0.056 mg/kg fentanyl alone. Tail-withdrawal latencies were redetermined 10, 30, and 100 min

after fentanyl administration unless emergence of respiratory depression required rescue with additional naltrexone treatments. These experiments were generally conducted twice per week.

Data Analysis

Drug effects were expressed as %Maximum Possible Effect (%MPE) using the following equation:

$$\%MPE = \left({(Test\ latency - Baseline\ latency)}\Big/{(20 - Baseline\ latency)}\right) * 100$$

where test latency was the tail-withdrawal latency from either 50°C or 54°C water obtained after drug administration, and baseline latency was the latency from either 50°C or 54°C water obtained before drug administration. Maximum antinociceptive effects were also determined for each drug or mixture at the group mean and individual level for each thermal stimulus intensity. Maximum effect was defined as the highest effect produced by any dose. Group mean maximum effects were compared using a one-way repeated-measures analysis of variance, and a Tukey post-hoc test was conducted following a significant main effect. In addition, maximum effect values were used in the analysis described in the next paragraph.

Theoretically, fentanyl/naltrexone mixtures should be useful to generate precise increments in efficacy that can be used 1) to generate mixtures with efficacies not available in existing single molecules, 2) to calibrate efficacy requirements for drug effects in different procedures, and 3) to infer efficacies of other drugs tested in those procedures. For example, if the relative efficacies of naltrexone and fentanyl are set arbitrarily at 0 and 1, respectively, then mixtures of 1:3, 1:1 and 3:1 fentanyl/naltrexone (after correcting for ligand affinity) will have relative efficacies along this continuum of 0.25, 0.5, and 0.75 respectively (i.e. relative efficacy = fractional contribution of fentanyl to the total drug in the mixture). The efficacy requirement of a

given procedure can then quantified by (a) testing effects of fentanyl and naltrexone alone and of all three mixtures, (b) generating efficacy-effect functions to relate maximum effects of each drug and mixture to the fentanyl proportion and associated relative efficacy, and (c) using nonlinear regression to determine the EP_{50} value, defined as the "effective proportion" of fentanyl to produce a maximum effect equal to 50% MPE in that procedure. EP_{50} values can then be compared across procedures. Additionally, once the efficacy-effect relationships are established, efficacy of a test drug can then be estimated as the fentanyl proportion that produces maximum effects equivalent to that of the test drug. To evaluate the utility of this approach, efficacy-effect curves were generated using nonlinear regression (GraphPad Prism, La Jolla, CA) to fit maximum effects data for fentanyl alone, naltrexone alone, and each mixture at each temperature using the equation:

$$Effect = 100 \times {(fentanyl\ proportion^{Hill\ Slope})} \Big/ {(EP50^{Hill\ Slope} + (fentanyl\ proportion^{Hill\ Slope}))}$$

where fentanyl proportion was the fractional contribution of fentanyl to the total drug in the mixture, and EP_{50} was the fentanyl proportion that produced a maximum effect equivalent to 50% maximum possible effect. Relative efficacies of test compounds were then estimated for each individual monkey by comparing maximum effects of each drug at each temperature with the group mean efficacy-effect curves. Specifically, relative efficacy was defined as the fentanyl proportion at which maximum effects of the test drug deviated least from the efficacy-effect functions. Deviation was quantified as the sum of the differences between test drug maximum effect and efficacy-effect curve at both 50 and 54°C, and the fentanyl proportion was identified at which deviation was smallest. Individual test drug values were then averaged to yield group

mean values and these data were analyzed using a one-way repeated measures analysis of variance. In the presence of a significant main effect, comparisons between test drug maximum effects were made using the Tukey's test.

For pretreatment and time course studies, two-way repeated-measures analysis of variance was performed with experimental manipulation (e.g. pretreatment) and fentanyl dose or time after fentanyl administration as the main independent variables. Following a significant interaction, a Holm-Sidak post-hoc test was performed, and the criterion for significance was $p<0.05$. Naltrexone pA_2 values were determined as described previously (Bowen et al., 2002).

Drugs

Fentanyl HCl, (−)-naltrexone HCl, morphine sulfate, and (−)-oxycodone HCl were supplied by the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). (−)-Nalbuphine HCl was provided by Dr. Kenner Rice (Drug Design and Synthesis Section, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD). (±)-Methadone HCl and (±)-buprenorphine HCl were purchased from Spectrum Chemicals (Gardena, CA). NAQ HCl was synthesized and provided by Dr. Yan Zhang (Li et al., 2009). Fentanyl, naltrexone, buprenorphine, nalbuphine, oxycodone, morphine, methadone, and all mixtures were dissolved in sterile water. NAQ was dissolved in 50% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and 50% sterile water. All drug doses were expressed as the salt forms listed above, and administered intramuscularly in the thigh.

RESULTS

Fentanyl-Naltrexone fixed-proportion mixtures

Across all baseline sessions before drug administration, monkeys always left their tail in 38°C water for 20 s, and the mean tail withdrawal latencies at 50°C and 54°C were 1.1 ± 0.5 s and 0.7 ± 0.1 s, respectively. Figure 2 left panels show the antinociceptive effects of fentanyl alone and following fixed naltrexone dose (0.0032-0.032 mg/kg, IM) pretreatments at 50°C (top) and 54°C (bottom). Fentanyl alone produced dose-dependent and full (≥90%MPE) antinociception at both temperatures in all monkeys. Increasing naltrexone dose pretreatments produced parallel rightward shifts in the fentanyl dose-effect function at both temperatures. Mean fentanyl ED_{50} values are shown in Table 3, and the naltrexone pA_2 values (95% confidence limits) were 8.58 (8.35, 8.82) and 8.50 (7.96, 8.52) for 50°C and 54°C, respectively. Figure 2 right panels show the antinociceptive effects of fentanyl alone, naltrexone alone, and the three fentanyl/naltrexone mixtures at 50°C (top) and 54°C (bottom). Maximum effect values from mean dose-effect curves are shown in Table 1, and maximum effect values in individual monkeys are shown in Table 2. As in the left panels, fentanyl alone produced dose-dependent antinociception, whereas naltrexone alone was ineffective at both temperatures (<5% MPE). Fentanyl/naltrexone mixtures produced a naltrexone proportion-dependent decrease in maximum effects. Fentanyl alone and the 1:0.025 fentanyl/naltrexone mixture produced maximum effects that were significantly different from both naltrexone alone and the 1:0.22 fentanyl/naltrexone mixture at both 50°C and 54°C (50°C: $F_{1.5,4.4}$=16.0, p=0.0111; 54°C: $F_{1.9,5.7}$=31.3, p=0.0009).

MOR ligands

Figure 3 shows the antinociceptive effects of the MOR ligands NAQ, nalbuphine, buprenorphine, oxycodone, morphine, and methadone at both 50°C (top) and 54°C (bottom).

Maximum effects values from mean dose-effect curves are shown in Table 1, and maximum effect values in individual monkeys are shown in Table 2. All drugs except NAQ produced maximum or near maximum antinociceptive effects at 50°C, and the rank of order of maximum effects at 54°C (from lowest to highest) was NAQ, buprenorphine, nalbuphine, morphine, oxycodone and methadone. In general, the sensitivity of individual monkeys to declining efficacy of fentanyl/naltrexone mixtures paralleled sensitivity to declining efficacy of test compounds. For example, the 1:0.22 fentanyl/naltrexone mixture produced the greatest antinociceptive effect at 54°C (44.2%MPE) in M1478, and this monkey also displayed the greatest or close to the greatest maximum individual antinociceptive effect at 54°C following nalbuphine, buprenorphine, or NAQ administration. In contrast, the 1:0.074 fentanyl/naltrexone mixture produced the least antinociceptive effect at 54°C in M1503, and this monkey also showed the weakest or close to the weakest individual antinociceptive effects at 54°C following nalbuphine, buprenorphine, or NAQ administration.

Efficacy estimates of MOR ligands relative to fentanyl and naltrexone

Figure 4A shows efficacy-effect curves that relate $\%MPE_{max}$ effects of fentanyl, naltrexone, and each mixture at 50 and 54°C to the proportion of fentanyl in the mixture from 0 (naltrexone alone) to 1 (fentanyl alone). Comparison of the nonlinear fits for the two different temperatures using the extra sum-of-squares F-test demonstrated that each temperature data set was best fit by different nonlinear functions ($F_{2,6}$=7.3, p=0.0249). For 50°C, the hill slope was 4.26, EP_{50} value (95% confidence limits) was 0.39 (0.34, 0.46), and R^2 was 0.99. For 54°C, the hill slope was 6.66, EP_{50} value was 0.53 (0.41, 0.61), and R^2 was 0.98. The 95% confidence limits for the EP_{50} values at 50 and 54°C overlapped. Figure 4B shows the best fit for the

maximum effect of each test drug to the efficacy-effect curves defined by the naltrexone-to-fentanyl continuum. Using this analysis, the efficacy of each compound relative to the naltrexone-to-fentanyl continuum was determined and results reported in Table 4. Comparison of maximum effects demonstrated that fentanyl and methadone both produced significantly higher maximum effects compared to buprenorphine, NAQ, and naltrexone ($F_{2.3, 6.8}$=23.3, p=0.0008). In addition, buprenorphine also produced significantly higher maximum effects compared to NAQ.

Effects of NAQ or fentanyl/naltrexone (1:0.22) pretreatment

Figure 5 shows cumulative fentanyl dose-effect functions alone or following a 15-min pretreatment with the low-efficacy MOR ligand NAQ (10 mg/kg; left panels) or the low-efficacy 1:0.22 fentanyl/naltrexone mixture (0.032 mg/kg; right panels) at both 50°C (top) and 54°C (bottom) thermal intensities, and fentanyl ED50 values are shown in Table 3. Consistent with the results described in Figure 2, fentanyl alone produced dose-dependent antinociception at both thermal intensities. NAQ pretreatment produced a significant (~9-fold) increase in the fentanyl ED_{50} value at 54°C (Table 3) and significantly attenuated the antinociceptive effects of cumulative 0.032 mg/kg fentanyl (fentanyl dose: $F_{2,6}$=566.3, p<0.0001; NAQ: $F_{1,3}$=176, p=0.0009; interaction: $F_{2,6}$=367.1, p<0.0001). Conversely, pretreatment with 0.032 mg/kg 1:0.22 fentanyl/naltrexone did not significantly increase in the fentanyl ED_{50} value at 54°C (Table 3), although it did significantly decrease the antinociceptive effects of cumulative 0.032 mg/kg fentanyl at 54°C (fentanyl dose: $F_{2,6}$=24.4, p=0.0013; interaction: $F_{2,6}$=16.1, p=0.0038).

Time course as a factor in drug-interaction studies

Figure 6 shows the time course of antinociception produced at 50 and 54°C by 0.056

mg/kg fentanyl administered in combination with 0.0041 mg/kg naltrexone. When these two doses were administered simultaneously (i.e. 0.056 mg/kg of the 1:0.074 fentanyl/naltrexone mixture), submaximal antinociceptive effects were observed at both 50 and 54°C, and these effects dissipated after 30-100 min. The effects of this bolus mixture after 10 min were similar to the effects observed when the same dose of this mixture was tested as part of the cumulative dose-effect curve (from Figure 2). Additionally, the effects of this bolus mixture dose were similar to effects observed with the fentanyl dose was administered three min after the naltrexone dose. However, when fentanyl was administered three min before naltrexone, the experiment had to be terminated because of severe sedation and respiratory depression in two monkeys that required additional naltrexone administration.

Discussion

The primary aim of the present study was to evaluate the degree to which fixed-proportion mixtures of fentanyl and naltrexone would produce effects predicted by receptor theory for mixtures of a competitive reversible agonist and antagonist targeting a common receptor. A secondary aim was to evaluate the utility of results with fentanyl/naltrexone mixtures for establishing an efficacy-effect scale that could be used to quantify (a) efficacy requirements for different drug effects, and (b) relative efficacies of different MOR ligands. There were three main findings. First, as predicted by receptor theory, the addition of naltrexone to fentanyl produced a naltrexone proportion-dependent decrease in the maximal antinociceptive effects of fentanyl/naltrexone mixtures. Second, the proportion of fentanyl in the mixtures served as a metric for efficacy of the mixtures, and this scale provided a strategy for quantifying efficacy requirements for different drug effects (i.e. antinociception at 50°C vs. 54°C) and relative in vivo

efficacies of different MOR ligands. Lastly, the results reported here also provide insight into factors that can limit utility of this approach. Overall, these results support the potential use of agonist/antgonist mixtures as tools in basic research, while also suggesting factors that may influence the usefulness of this approach.

Fentanyl alone produced dose- and thermal intensity-dependent antinociception in rhesus monkeys, whereas naltrexone alone produced <10%MPE up to the largest doses tested. These results were consistent with a large body of literature demonstrating the antinociceptive effects of fentanyl in humans (Finch and DeKornfeld, 1967), nonhuman primates (Nussmeier et al., 1991; Gatch et al., 1995; Maguire and France, 2014) and rodents (Millan, 1989; Walker et al., 1994; Minami et al., 2009). Because naltrexone failed to produce significant antinociception, one method to determine whether a behaviorally active dose range was administered would be to give naltrexone as a pretreatment to cumulative fentanyl. In this experiment, receptor theory would predict that increasing naltrexone fixed-dose pretreatments would produce parallel rightward shifts in the fentanyl dose-effect function. The present results were consistent with this hypothesis, and the naltrexone pA_2 values reported in this study were consistent with previous naltrexone studies in monkeys (Rowlett et al., 2000; Bowen et al., 2002; Gerak and France, 2007). Overall, these results provide an empirical foundation to interpret the antinociceptive effects of fixed-proportion fentanyl and naltrexone mixtures.

Receptor theory predicts that fixed-proportion mixtures of a competitive reversible agonist and antagonist should produce maximal effects that decline as the proportion of agonist in the mixture declines. Results support this prediction. Specifically, the MOR agonist fentanyl produced dose-dependent antinociception at both 50 and 54°C, and mixtures of fentanyl with the MOR antagonist naltrexone produced decreasing maximal antinociceptive effects as the

proportion of fentanyl in the mixture decreased. The declining maximal effects of fentanyl/naltrexone mixtures with declining fentanyl proportions resembles the declining maximal effects of mu agonists produced by pretreatments with irreversible antagonists (Zernig et al., 1994; Walker and Young, 2002). As such, fixed-proportion mixtures with competitive antagonists may serve as an alternative to use of irreversible antagonists for research on the role of efficacy as a determinant of drug effects. This approach may be especially useful in research on systems for which competitive antagonists are available, but irreversible antagonists are not.

Because the agonist/antagonist proportion determined the apparent efficacy of a mixture, this proportion could be used as a quantitative measure of in vivo efficacy. In the present study, this metric was applied in two ways. First, we evaluated the efficacy requirements for antinociception at 50 and 54°C by comparing the fentanyl proportion required to produce a maximal effect of 50% MPE at each temperature. Although the 95% confidence limits for these values overlapped, the higher mean value at 54°C agrees with other data to suggest that efficacy requirements for antinociception are higher at 54°C than 50°C (Walker et al., 1993; Banks et al., 2010b; Maguire and France, 2014). Additionally, although this study compared efficacy requirements of similar endpoints (i.e. antinociception at two different stimulus intensities in rhesus monkeys), it is theoretically possible to apply this approach across multiple endpoints that could include not only other behavioral and physiological endpoints in rhesus monkeys, but also endpoints in other species or in in vitro assays. For example, two undesirable effects of MOR agonists that limit their clinical utility are respiratory depression and abuse liability, and fentanyl/naltrexone mixtures could be used to quantify the efficacy requirement for each of these or any other MOR agonist effect of interest. These experiments would also provide empirical

data on the utility of agonist/antagonist mixtures to assess the efficacy requirements of different experimental endpoints.

A second implication of the present study was that fentanyl/naltrexone mixtures could be used to stratify MOR ligands based on their in vivo antinociceptive efficacy in rhesus monkeys. In the present study, NAQ produced < 10%MPE and these results are consistent with and extend previous findings in mice (Zhang et al., 2014; Yuan et al., 2015) and rats (Siemian et al., 2016). Buprenorphine (Walker et al., 1995; Maguire and France, 2014), nalbuphine (Walker et al., 1993; France and Gerak, 1994; Banks et al., 2010b), morphine (Bowen et al., 2002), oxycodone, and methadone (Stevenson et al., 2003; Banks et al., 2010b) produced dose- and thermal intensity-dependent antinociception in the present study, and these results were generally consistent with the extant literature examining MOR agonists in a warm-water tail-withdrawal procedure in monkeys. With one major exception (see below regarding nalbuphine), the order of MOR efficacies for these drugs as ranked here agrees with the order of efficacies as determined by in vitro approaches such as agonist-stimulated GTPγS binding (Selley et al., 1998; Alt et al., 2001; Yuan et al., 2015). Specifically, both approaches yield a rank order of lowest-to-highest efficacy of naltrexone < NAQ < buprenorphine < morphine < oxycodone < fentanyl < methadone. By comparing effects of these mu agonists to effects of fentanyl/naltrexone mixtures, it was possible not only to rank order drug efficacies, but also to provide a quantitative measure of those relative efficacies, expressed as fentanyl proportion.

Results with nalbuphine in the present study did not agree with previous in vitro results using agonist-stimulated GTPγS binding with either mouse MOR (Selley et al., 1998) or rat MOR (Alt et al., 2001). The basis for this difference between published GTPγS results and antinociceptive efficacy in rhesus monkeys remains to be empirically determined. Although there

are no published GTPγS results with any MOR ligand using monkey MOR, two lines of evidence support the conclusion that nalbuphine functions as a higher efficacy MOR ligand than buprenorphine in rhesus monkeys. First, in HEK cells expressing MOR and examining inhibition of forskolin-stimulated cAMP accumulation, nalbuphine produced similar efficacy to morphine (Gharagozlou et al., 2003). Second, the present nalbuphine results demonstrating greater antinociceptive effects of nalbuphine compared to buprenorphine are generally consistent with previously published studies in nonhuman primates (Walker et al., 1993; Walker et al., 1995; Maguire and France, 2014). In addition to these antinociceptive studies, nalbuphine also shows higher efficacy than buprenorphine in an assay of schedule-controlled responding. For example, nalbuphine produced dose-dependent and near complete suppression of operant responding (Stevenson et al., 2003; Banks et al., 2010b), whereas buprenorphine decreased operant responding to approximately 65% of control (Negus et al., 2002). Overall, the present results highlight potential species differences in MOR ligand efficacy and support the utility of nonhuman primates in preclinical pharmacology research.

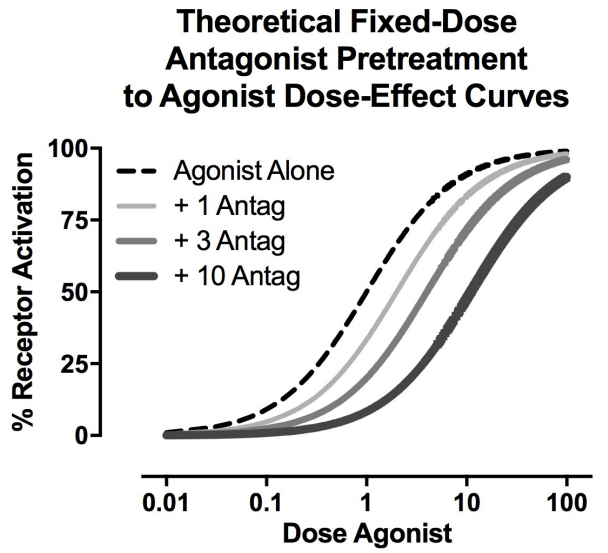
Although the present results support the concept that agonist/antagonist mixtures can be used manipulate apparent in vivo efficacy, these results also revealed factors that can influence the precision of this approach. Two particular limitations will be mentioned here. First, the efficacies of the constituent drugs in a mixture define the upper and lower boundaries of efficacy that can be assessed. For example, in the present study, fentanyl served as the agonist, and studies of in vitro agonist-stimulated GTPγS binding suggest that some MOR ligands (e.g. methadone) may have higher efficacy than fentanyl (Selley et al., 1998; Alt et al., 2001). Because fentanyl defines the upper boundary of efficacy than can be achieved with fentanyl/naltrexone mixtures, these mixtures would not be useful for scaling effects of drugs like

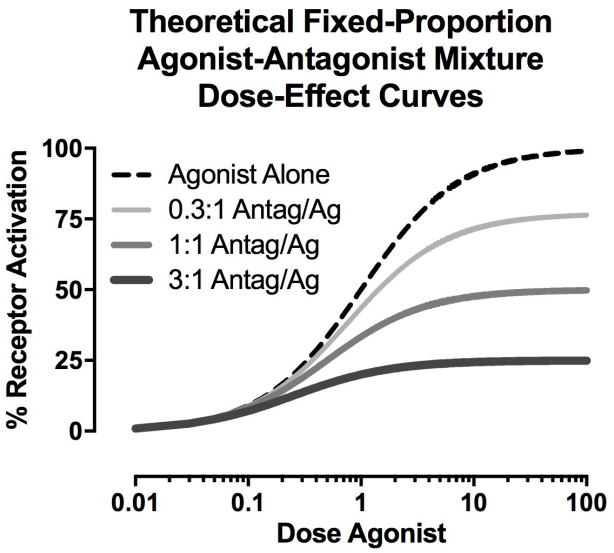
methadone that may have higher efficacy than fentanyl. Similarly, these mixtures would not be useful for scaling effects of drugs that have lower efficacy than naltrexone.

Second, although agonist/antagonist proportions can be precisely controlled in a mixture, the pharmacokinetics and associated time courses of the constituent drugs play a key role in determining the proportional drug concentrations at receptor targets after a in vivo drug administration. For example, in the present study, cumulative administration of the 1:0.074 fentanyl/naltrexone mixture could be safely studied at doses up to 0.32 mg/kg. However, bolus administration of this mixture at dose of 0.1 mg/kg fentanyl + 0.0074 mg/kg naltrexone could not be studied due to the onset of severe sedation and respiratory depression in at least one monkey. This suggests that, after bolus administration, fentanyl distributes more quickly than naltrexone to receptors that mediate sedation and respiratory depression. This difference may be mitigated during cumulative dosing by sustained effects of naltrexone doses administered early in the dosing regimen. Additionally, the impact of these pharmacokinetic issues may be influenced by both the agonist/antagonist proportion and overall mixture dose. For example, in the present study, both cumulative and bolus administration of 0.056 mg/kg 1:0.074 fentanyl/naltrexone produced similar effects. However, administration of fentanyl just three min before naltrexone resulted in severe sedation and respiratory depression. Overall, these results highlight time course of drug effects as a key consideration in the deployment of competitive agonist/antagonist mixtures for both basic research or clinical studies.

As a final note, the present results with fentanyl/naltrexone mixtures can be compared to development of opioid formulations that include a MOR agonist in combination with the competitive reversible antagonist naloxone (e.g. fixed-proportion formulations of oxycodone + naloxone or buprenorphine + naloxone) (Mendelson and Jones, 2003; Chen et al., 2014; Fanelli

and Fanelli, 2015; O'Brien, 2015). Consumption of these products by intended enteral routes of administration results in naloxone distribution to the gastrointenstinal tract (which may reduce constipating effects of the agonist), but limited distribution to the central nervous system due to extensive first-pass metabolism by the liver (resulting in limited interference with centrally mediated agonist effects). However, parenteral administration bypasses first-pass metabolism, resulting in greater naloxone distribution to the central nervous system and potential blockade of centrally mediated agonist effects and/or precipitation of withdrawal in opioid-dependent subjects. As a result of these characteristics, naloxone combination products are thought to have fewer gastrointestinal side effects and lower abuse liability than the agonists alone. The experimental design deployed in the present study could be used to test this hypothesis, with the caveat that naloxone's relatively short duration of action may hamper naloxone's utility for this type of research. For example, naloxone should be more potent to produce proportion-dependent downward shifts in agonist dose-effect curves for gastrointenstinal than centrally-mediated effects after enteral but not parenteral administration. The present study also suggests how the general concept of agonist+antagonist mixtures can be expanded beyond naloxone-containing combination products to include other antagonists such as naltrexone, or agonist+antagonist mixtures targeting other receptors, yielding mixtures with other pharmacological profiles.





$$\%\,RA = f\{R_t \bullet E_A \bullet \left(\frac{A}{A+K_{DA}(1+(B/K_{DB}))}\right)\}$$

$$\%\,RA = f\{R_t \bullet E_A \bullet \left(\frac{A}{A+K_{DA}(1+(pA/K_{DB}))}\right)\}$$

% RA = % Receptor Activation
A = Agonist Dose
f = Transduction function
R_t = Receptor Number
E_A = Agonist Efficacy
K_{DA} = Agonist Affinity

B = Antagonist Dose
p = Antagonist/Agonist Proportion (B/A)
K_{DB} = Antagonist Affinity

All doses in K_D units

Figure 1

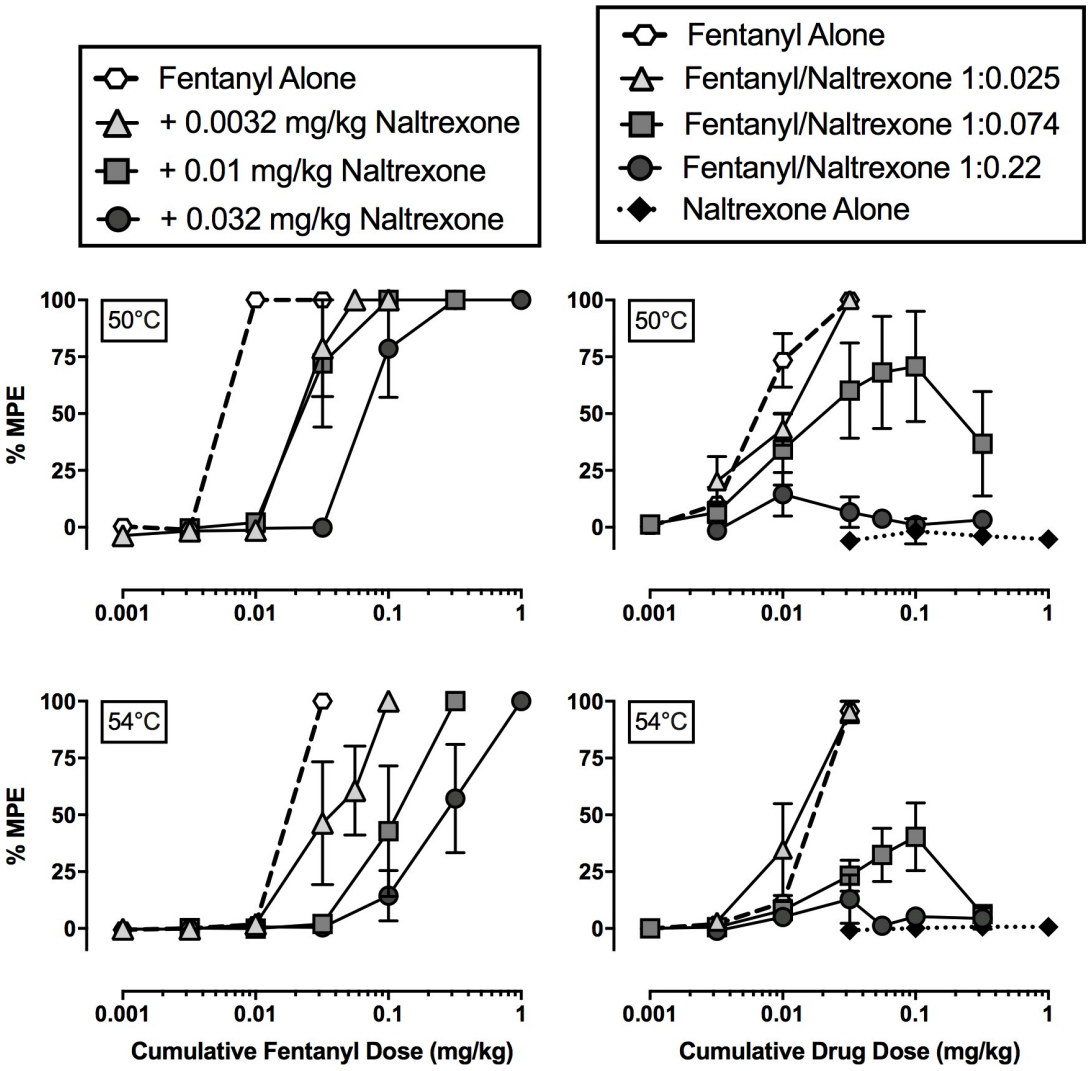


Figure 2

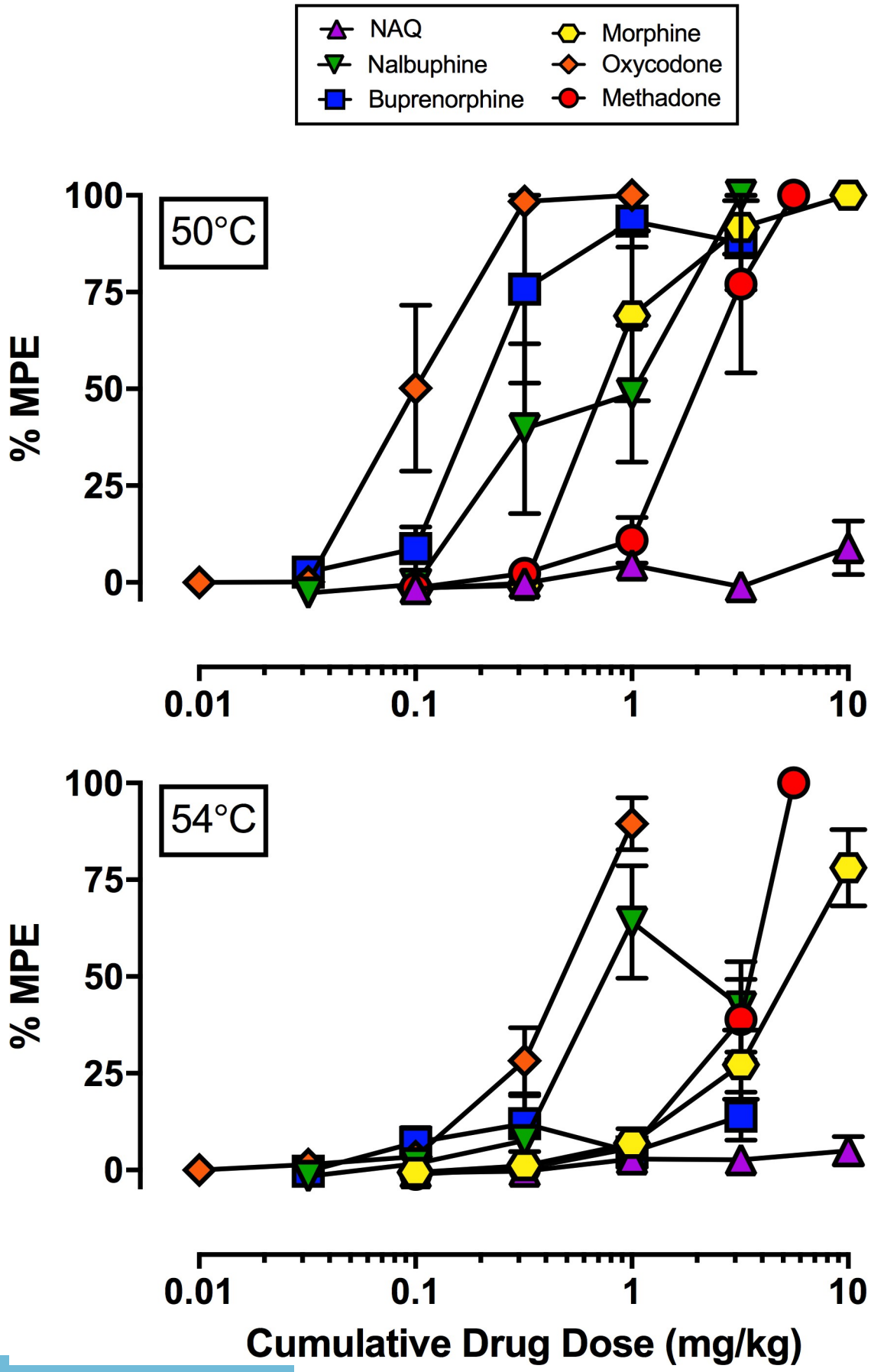


Figure 3

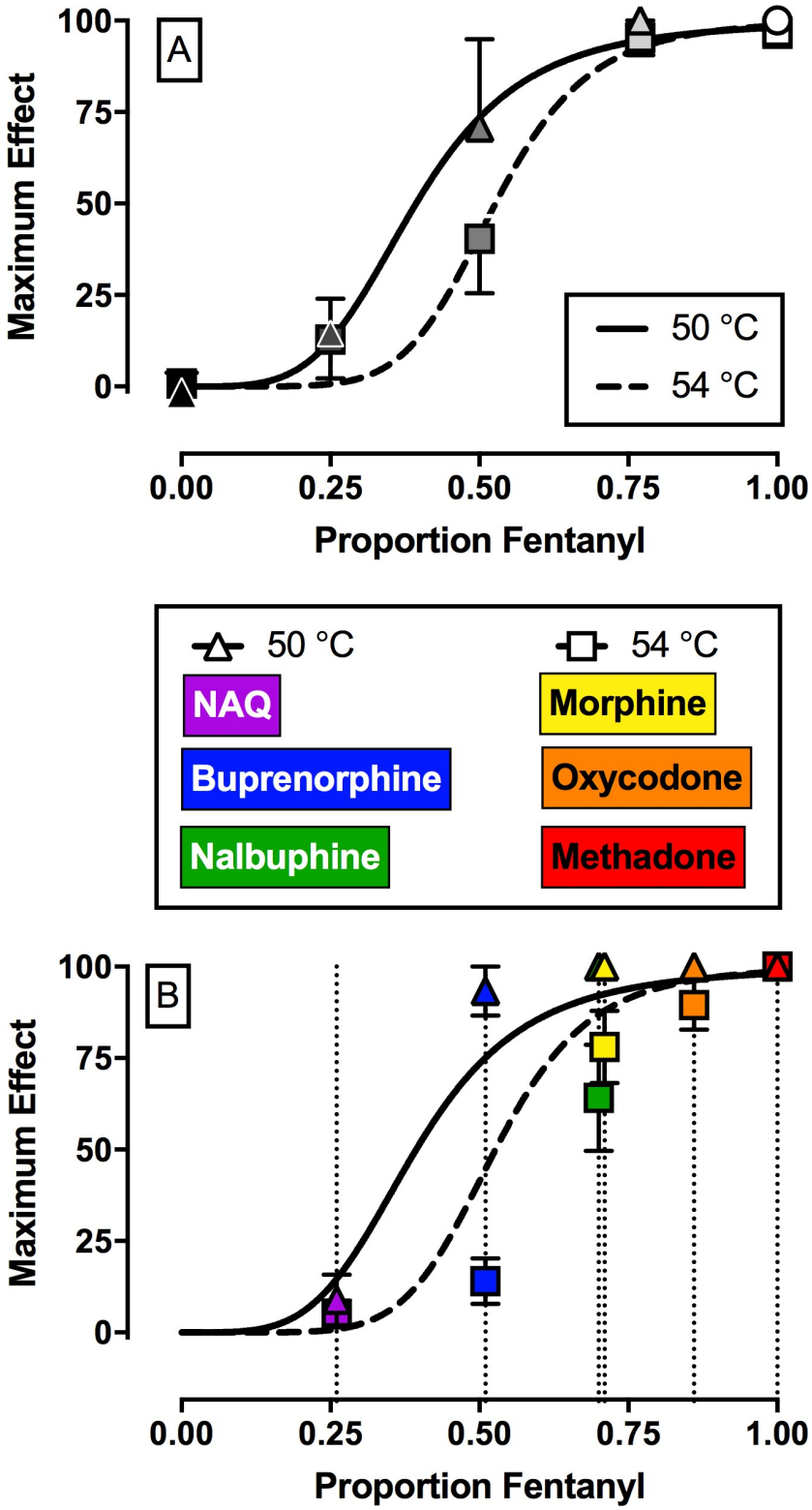


Figure 4

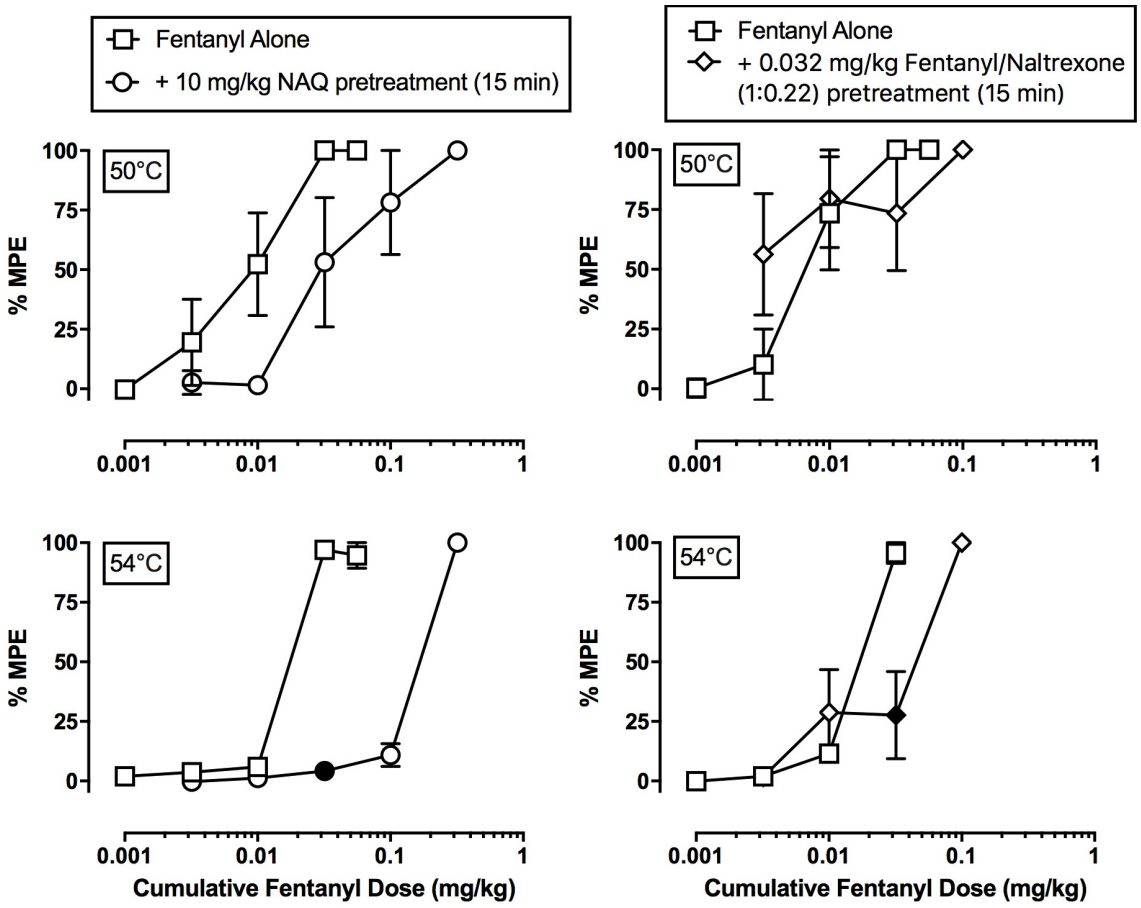


Figure 5

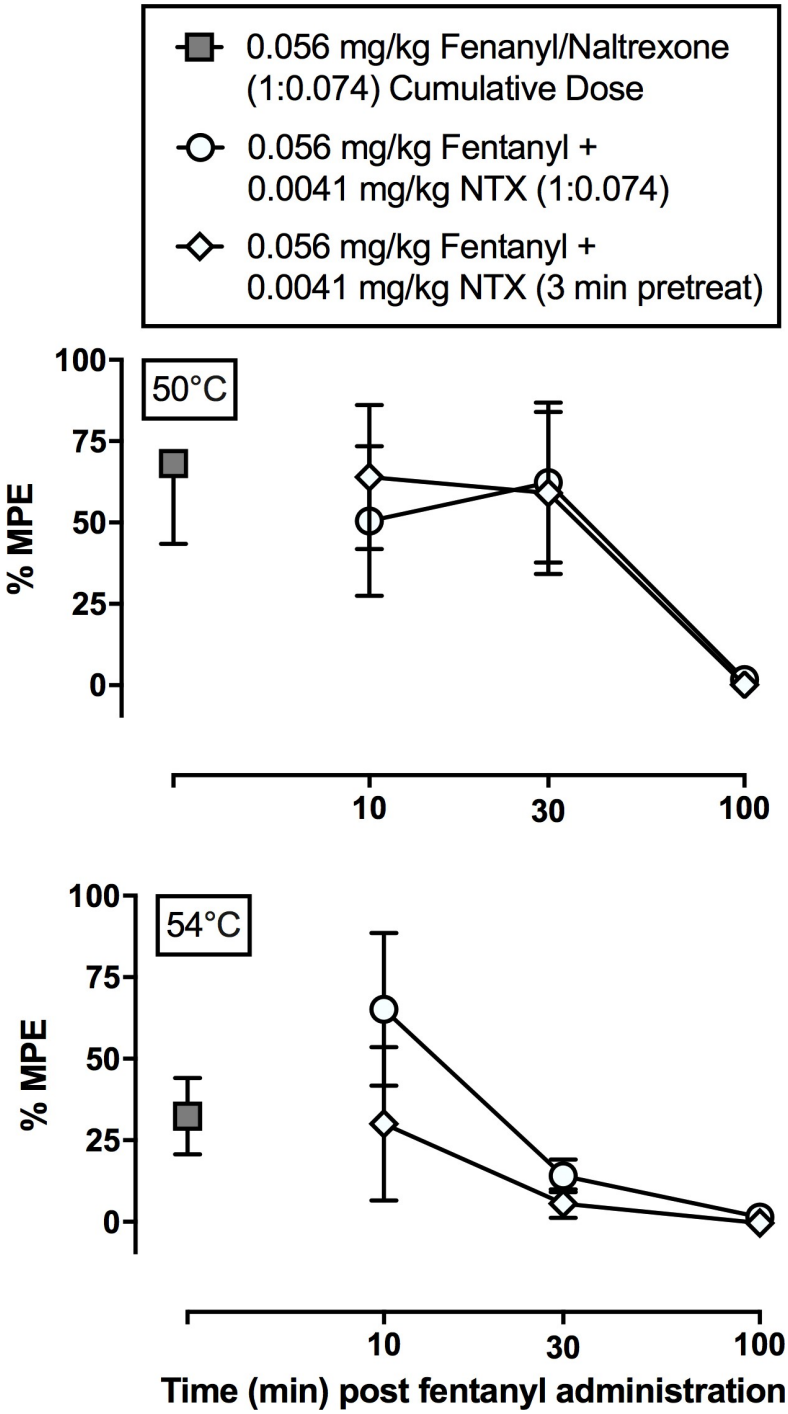


Figure 6

Figure Legends

Figure 1: Theoretical curves simulated from the Furchgott equation for receptor theory (Ruffolo, 1982). Left panel shows rightward shifts in a competitive reversible agonist dose-effect function after pretreatment with increasing fixed doses of a competitive reversible antagonist. Right panel shows downward shifts in a competitive reversible agonist dose-effect function when agonist and antagonist are co-administered in fixed-proportion mixtures. Equations and definition of terms are shown below the panels. For this simulation, agonist dose A and antagonist dose B vary in K_D units (i.e. at a dose of 1, dose = K_D); R_t was set arbitrarily at 100, and all other variables were set arbitrarily at 1. Note that in the left panel, antagonist dose is a fixed dose B that remains constant across a range of agonist doses. For the right panel, antagonist dose is a fixed proportion p of the agonist dose A, such that B=pA and increases in agonist dose are accompanied by increases in antagonist dose.

Figure 2: Effects of fixed-dose naltrexone pretreatments to fentanyl and fixed-proportion fentanyl/naltrexone mixtures in an assay of thermal nociception in male rhesus monkeys. Left panels show effects of fentanyl alone and after increasing naltrexone doses administered as a 15-min pretreatment to fentanyl at 50°C (top) and 54°C (bottom) thermal intensities. Right panels show effects of fentanyl alone, naltrexone alone, and three fentanyl/naltrexone mixtures at 50°C (top) and 54°C (bottom). Abscissae: cumulative intramuscular fentanyl dose (left panels) or cumulative drug dose (right panels) in mg/kg. Note that for data with fentanyl/naltrexone mixtures in the right panels, the abscissa shows the fentanyl dose in the mixture, and the naltrexone dose = fentanyl dose x naltrexone proportion. Ordinates: % maximum possible effect (%MPE). All points represent mean ± SEM of 4 monkeys.

Figure 3: Effects of six different MOR ligands in an assay of thermal nociception in male rhesus monkeys. Top panel shows effects of NAQ, nalbuphine, buprenorphine, morphine oxycodone, and methadone at 50°C, and bottom panel shows effects at 54°C. Abscissae: cumulative intramuscular drug dose (mg/kg). Ordinates: % maximum possible effect (%MPE). All points represent mean ± SEM of 4 monkeys.

Figure 4: Top panel (A) shows maximum antinociceptive effect at 50°C (triangles) and 54°C (squares) as a function of the fentanyl proportion in the fentanyl/naltrexone mixture in male rhesus monkeys. Bottom panel (B) shows empirically determined maximum antinociceptive effects of NAQ, buprenorphine, nalbuphine, morphine, oxycodone, and methadone. Results were fit to the model generated from the top panel, and relative efficacy of each ligand was estimated as the fentanyl proportion to produce maximum effects at 50 and 54°C most like the test ligand. Abscissae: Efficacy expressed as Proportion Fentanyl. "0" denotes naltrexone alone, "1" denotes fentanyl alone, and the efficacy of each mixture (Emix) was calculated as the fractional contribution of fentanyl to the mixture as described in Methods. Ordinates: Maximum Effect. All points represent mean ± SEM of 4 monkeys.

Figure 5: Effects of cumulative fentanyl (0.001-0.32 mg/kg, IM) administered either alone or following a 15-min pretreatment with either 10 mg/kg NAQ (left panels) or 0.032 mg/kg fentanyl/naltrexone (1:0.22) (right panels) in rhesus monkeys. Abscissae: cumulative intramuscular fentanyl dose (mg/kg). Ordinates: % maximum possible effect (%MPE). All points represent mean ± SEM of 4 monkeys. Filled points denote statistical significance ($p<0.05$) compared to fentanyl alone.

Figure 6: Time course of antinociceptive effects of 0.056 mg/kg fentanyl in combination with 0.0041 mg/kg naltrexone administered simultaneously as a bolus dose of the 1:0.074 fentanyl/naltrexone mixture or with the naltrexone dose administered as a 3-min pretreatment to the fentanyl dose in rhesus monkeys. Antinociceptive effects of cumulative 0.056 mg/kg 1:0.074 fentanyl/naltrexone from Figure 2 are also plotted for comparison. Abscissae: fentanyl dose (mg/kg). Ordinate: % maximal possible effect (%MPE). Each point represents mean ± SEM of 4 monkeys.

Table 1: Group mean %MPE_{max} values and (±SEM) for each fentanyl/naltrexone combination or test drug administered in an assay of thermal nociception at 50°C and 54°C in rhesus monkeys (n=4).

Drug or Drug Mixture	%MPE_{max} (SEM)	
	50°C	**54°C**
Fentanyl	100 (0) *,†	96.4 (2.8) *,†
1:0.025 Fentanyl/Naltrexone	100 (0) *,†	95.3 (4.7) *,†
1:0.074 Fentanyl/Naltrexone	70.8 (24.2)	40.4 (14.9)
1:0.22 Fentanyl/Naltrexone	14.5 (9.5)	12.9 (10.7)
(−)-Naltrexone	-1.8 (5.6)	0.9 (0.4)
(±)-Methadone	100 (0)	100 (0)
(−)-Oxycodone	100 (0)	89.5 (6.7)
(−)-Morphine	100 (0)	78.1 (9.9)
(−)-Nalbuphine	100 (0)	64.1 (14.5)
(±)-Buprenorphine	93.3 (6.7)	14.0 (6.0)
NAQ	8.9 (6.9)	5.1 (3.7)

* Significantly different from Naltrexone ($p < 0.05$)

† Significantly different from 1:0.025 Fentanyl/Naltrexone mixture ($p < 0.05$)

Table 2: Individual $\%MPE_{max}$ values for each fentanyl/naltrexone combination or test drug administered in an assay of thermal nociception at 50°C and 54°C in rhesus monkeys.

	%MPE max							
	50° C				54° C			
Drug or Drug Mixture	M1414	M1473	M1478	M1503	M1414	M1473	M1478	M1503
Fentanyl	100	100	100	100	87	100	100	100
1:0.025 Fentanyl/Naltrexone	100	100	100	100	81.1	100	100	100
1:0.074 Fentanyl/Naltrexone	84.2	100	100	3.3	37.6	62.7	62.2	4.7
1:0.22 Fentanyl/Naltrexone	9	6.6	42.2	10.4	18.1	10	44.2	1.1
(−)-Naltrexone	9.5	2.1	-12.6	-1.6	3.2	1.8	-0.3	0.6
(±)-Methadone	100	100	100	100	100	100	100	100
(−)-Oxycodone	100	100	100	100	71.4	100	86.1	100
(−)-Morphine	100	100	100	100	74.9	52.8	54.7	100
(−)-Nalbuphine	100	100	100	100	52.8	100	71.9	31.7
(±)-Buprenorphine	73.2	100	100	100	32.1	18.7	29.3	16.5
NAQ	4.6	5.9	28.8	8	6.4	1.1	16.1	2.8

Table 3: Fentanyl ED_{50} values and (95% confidence limits) administered alone or following a 15-min pretreatment with naltrexone (0.0032-0.032 mg/kg), 10 mg/kg NAQ, or 0.032 mg/kg fentanyl/naltrexone (1:0.22) mixture in an assay of thermal nociception at 50°C and 54°C. Data are presented as the mean of 3 monkeys for the naltrexone pretreatment studies and mean of 4 monkeys for the NAQ and fentanyl/naltrexone mixture pretreatment studies. ‡ denotes non-overlapping 95% confidence limits (CL).

	ED_{50} in mg/kg (95% CL)	
	50°C	**54°C**
Fentanyl alone	0.006 (0.006, 0.006)	0.018 (0.018, 0.018)
+ 0.0032 mg/kg Naltrexone	0.021 (0.016, 0.028) ‡	0.035 (0.018, 0.069)
+ 0.01 mg/kg Naltrexone	0.018 (0.013, 0.05) ‡	0.169 (0.057, 0.228) ‡
+ 0.032 mg/kg Naltrexone	0.057 (0.043, 0.128) ‡	0.257 (0.109, 0.608) ‡
Fentanyl alone	0.006 (0.005, 0.006)	0.014 (0.012, 0.017)
+ 10 mg/kg NAQ pretreatment	0.041 (0.014, 0.118) ‡	0.155 (0.122, 0.197) ‡
Fentanyl alone	0.006 (0.005, 0.006)	0.014 (0.012, 0.017)
+ 0.032 mg/kg Fentanyl/Naltrexone (1:0.22) pretreatment	< 0.017 (0.002, 0.18) §	0.035 (0.015, 0.08)

§ ED50 value could only be determined in 2 out of 4 monkeys because no fentanyl dose produced < 50%MPE.

Table 4: Estimated efficacy of each compound relative to the naltrexone-to-fentanyl continuum in proportion fentanyl units (95% confidence limits) for each of the eight MOR ligands tested in rhesus monkeys (n=4). Individual $\%MPE_{max}$ values were fitted to the nonlinear function generated from the group mean results shown in Figure 4A.

Test Drug	Proportion Fentanyl (95% CL)
Fentanyl	0.94 (0.77, 1.12) ¶, #, Π
(±)-Methadone	1 (1, 1) ¶, #, Π
(−)-Oxycodone	0.86 (0.58, 1.13)
(−)-Morphine	0.71 (0.4, 1.02)
(−)-Nalbuphine	0.7 (0.37, 1.03)
(±)-Buprenorphine	0.51 (0.47, 0.54) #
NAQ	0.26 (0.16, 0.36)
(−)-Naltrexone	0.12 (-0.1, 0.33)

¶ Significantly different from Naltrexone ($p < 0.05$)

Significantly different from NAQ ($p < 0.05$)

Π Significantly different from Buprenorphine ($p < 0.05$)

Chapter 5: Discussion

This dissertation examined MOR agonist interactions on preclinical endpoints related to pain in nonhuman primates. There were three main findings. First, NMDA receptor antagonists failed to enhance the antiallodynic effects of either a moderate (nalbuphine) or high (oxycodone) efficacy MOR agonist. Second, the NOP receptor agonist Ro 64-6198 potentiated the antinociceptive effects of MOR agonists regardless of efficacy; however, the effect was far more modest than previously reported and was only observed under a narrow range of experimental conditions. Third, fixed-proportion MOR antagonist (i.e. naltrexone) and agonist (i.e. fentanyl) mixtures were successful in decreasing antinociceptive efficacy in an antagonist proportion-dependent manner. Overall, the results described in this dissertation add to the growing body of literature examining candidate opioid adjuncts to produce opioid-sparing effects by identifying pharmacological and experimental limitations of previously examined opioid interactions and in strengthening the case for employing complementary behavioral measures in opioid combination experiments. Furthermore, this dissertation provided several experimental design insights to guide future preclinical pain and candidate analgesic drug development research.

1. NMDA antagonist and opioid agonist interactions

The main finding of Chapter 2 was that the NMDA receptor antagonists ketamine and MK-801 failed to enhance the antiallodynic effects of either the moderate (i.e. nalbuphine) or high (i.e. oxycodone) efficacy MOR agonists. Furthermore, NMDA antagonist and MOR agonist combinations reduced the experimental therapeutic index of the MOR agonists to selectively produce antiallodynia vs. behavioral sedation as compared to the MOR agonists alone.

Generally, the experimental therapeutic index decreased as the amount of NMDA receptor antagonist in the mixture increased. This would suggest that increasing NMDA antagonist doses in fixed proportion combinations with MOR agonists would be more detrimental than producing clinically beneficial effects. These results further highlight (1) the utility of nonhuman primates in preclinical assessments of candidate analgesics and (2) the necessity of employing complementary assays to discern the selectivity of antinociceptive vs. sedative effects of putative MOR agonist combination medication therapies for clinical pain management.

The reported antinociceptive interactions between the NMDA receptor antagonists and MOR agonists were consistent with previous reports in rhesus monkeys (Banks et al, 2010) and humans (Lee and Lee, 2016). However, these results are inconsistent with a previous study in squirrel monkeys (Allen and Dykstra, 2001). The lack of a synergistic interaction in Chapter 2 results and those reported in Banks (2010) are in contrast to results reported by Allen and Dykstra (2001). These differences are most likely attributed to the noxious stimulus utilized in each experimental design. For example, a thermal noxious stimulus was utilized in both Chapter 2 and Banks (2010) whereas Allen and Dykstra (2001) utilized a mild electrical shock as the noxious stimulus. Interactions between NMDA receptor antagonists and MOR agonists have been previously shown to differentially affect antinociceptive responses depending upon stimulus modality. For example, in clinical laboratory experiments the NMDA receptor antagonist ketamine potentiated the antinociceptive effects of fentanyl against an electrical, but not thermal noxious stimulus (Tucker et al, 2005). Thus, stimulus modality appears to be a critical factor in the antinociceptive interactions between NMDA receptor antagonists and MOR agonists. These discrepancies may suggest further limitation to any potential clinical utility that NMDA antagonists and MOR agonists may have in the management of pain.

The reported antinociceptive interactions between NMDA receptor antagonists and MOR agonists in Chapter 2 were also inconsistent with several previous rodent studies examining MOR agonist and NMDA antagonist interactions (Baker, Hoffman, Meert, 2002; Holtman Jr et al, 2008). For example, dextromethorphan, ketamine, and ketamine metabolites (S(+)- and R(-)-norketamine) were previously shown to enhance the antinociceptive effects of MOR, but not KOR and DOR, agonists in rats across multiple pain states (Baker, Hoffman, Meert, 2002; Holtman Jr et al, 2008). Since the noxious stimuli didn't differ between Chapter 2 and the aforementioned rodent studies (e.g. warm-water tail withdrawal vs. hot plate, respectively), this apparent discrepancy suggests differences between rodents and nonhuman primates in antinociceptive effects mediated by NMDA receptor activity (i.e. neuroanatomical differences). Moreover, one of the major differences between the rodent studies and the experiments in Aim 1 is several of the NMDA receptor antagonists utilized (eg. dextromethorphan, S(+)- and (R-)-norketamine). A meta-analysis suggested that ketamine combinations with opioids for management of acute pain were not superior to the opioid alone and may exacerbate some undesirable side effects (Lee and Lee, 2016). Thus, the clinical utility of ketamine or other NMDA antagonists as an adjunct to MOR agonists for the management of pain is very unlikely. Moreover, ketamine-MOR combinations in several clinical trials lead to increased "neuropsychiatric" side effects in some patients (Lee and Lee, 2016). Overall, the results reported in this dissertation were consistent with conclusions from this meta-analysis and support the utilization of nonhuman primates and multi-modal preclinical dependent measures to develop candidate analgesics.

2. NOP agonist and opioid agonist interactions

The main finding of Chapter 3 was that the high efficacy NOP receptor agonist Ro 64-6198 (Jenck et al, 2000) potentiated the antinociceptive effects of MOR agonists regardless of efficacy; however, this effect was far more modest than previous reports, and was only observed to occur under a narrow range of experimental conditions. Furthermore, the previously reported antinociceptive effects of Ro 64-6198 alone in rhesus monkeys (Ko et al, 2009; Podlesnik et al, 2011; Cremeans et al, 2012) were not replicated at comparable or 3-fold higher doses (0.1 and 0.32 mg/kg IM, respectively).

The results indicating that NOP agonists did not impact our complementary undesirable MOR agonist effect add to the existing reports of buprenorphine and NOP agonist combinations and bivalent MOR-NOP agonists. Ro 64-6198 had been previously shown not to increase the potency or efficacy for buprenorphine to produce respiratory depression or pruritis (Cremeans et al, 2012). Furthermore, the mixed-action MOR-NOP agonist BU08028 showed no significant production of respiratory depression (compared to MOR agonist alone), and did not maintain drug-self-administration (Cremeans et al, 2012; Ding et al, 2016). While some of these undesirable effects may still persist at higher doses of buprenorphine+Ro 64-6198 combinations (i.e. abuse liability and respiratory depression), the increase in antinociceptive potency compared to buprenorphine alone may result in a decrease in the occurrence of these opioid-related adverse effects (Li, 2019). Overall, these data would support the utility of MOR+NOP combinations for clinical pain treatment.

One potential limitation of the clinical utility of NOP+MOR combinations is the lack of pharmacological selectivity as NOP activity may potentiate the undesirable effects of MOR agonists as well. The potentiation of both the antinociceptive effects and antagonism of rate-decreasing effects of nalfurafine by Ro 64-6198 at a single dose (0.01 and 0.001 mg/kg,

respectively) further complicate the translatability of NOP+MOR agonist combinations to clinical utility. These findings suggest that Ro 64-6198 potentiation is perhaps not selective for (1) mu-opioid receptor agonists and (2) opioid receptor-mediated antinociception. If this potentiation is not selective for MOR agonists, this could suggest another false-positive result as DOR and KOR agonists will produce antinociception in preclinical assays, but are not clinically useful analgesics. However, this could also suggest that mixed action MOR/KOR agonists (such as butorphanol) may be viable candidates for future NOP combinations.

3. Opioid adjuncts as a useful approach to produce an opioid-sparing effect

In comparison with interactions between MOR agonists and other candidate adjuncts targeting other receptors, the MOR agonist antinociceptive potentiation reported in this dissertation is much more modest (NOP-MOR) or nonexistent (NMDA-MOR). For a list of previous Mu-Plus interactions, see Table 1. For example, the serotonin uptake inhibitors fluoxetine and clomipramine potentiated the antinociceptive effects of MOR agonists in rats and rhesus monkeys, respectively, by nearly 10-fold (Nayebi et al, 2001; Banks et al, 2010). Furthermore, the cannabinoid receptor agonists CP 55,940 and Δ^9-THC both potentiated the antinociceptive effects of MOR agonists nearly 10-fold in both rhesus monkeys and rats (Maguire and France, 2014, 2018). Moreover, in some cases, DOR potentiation of MOR mediated antinociception was greater than reported in Chapters 2 and 3 experiments (e.g. SNC80 + methadone, fentanyl, or nalbuphine), but was roughly similar with other DOR-MOR combinations (e.g. SNC80 + morphine and MSF61 + fentanyl) (Stevenson et al, 2003; Negus et al, 2009). Additionally, the KOR agonist nalfurafine potentiated oxycodone antinociception in rodents to a greater degree, roughly 10-fold, than results reported in the Aim 1 and 2 experiments

(Townsend et al, 2017), and mixtures with the KOR agonist spiradoline and morphine or etorphine potentiated antinociceptive effects of the MOR agonist alone by roughly 10-fold and 5-fold, respectively (Minervini et al, 2018). Overall, the absence of NMDA antagonist enhancement of MOR agonist antinociception and the small magnitude of NOP agonist enhancement of MOR agonist antinociception compared to published literature with other potential adjuncts targeting other receptor systems do not support the further development of either NMDA antagonists as MOR agonist adjuncts but does support NOP agonists as candidate analgesics for the treatment of acute pain. However, it is unknown if NOP-MOR agonist combinations or bivalent ligands will be superior to MOR agonists alone in the production of analgesia. Finally, the results do provide insight into the applicability of opioid-combination experiments for considerations into the experimental designs of future MOR agonist combination medication development experiments.

Despite promising results from previous proposed adjunct combinations (eg. serotonin uptake inhibitors, cannabinoid receptor agonists, DOR agonists, KOR agonists, NOP agonists, imidazoline I2 receptor agonists) there does not seem to be much translational progress from preclinical results to clinical application of multiple molecule therapies. The increasing number of recent clinical trials suggests there seems to be more interest in one alternative to fixed-proportion drug mixtures: bivalent ligands that target multiple receptor systems of interest. For example, the NOP-MOR bivalent ligand cebranopadol has been investigated in a wide range of clinical trials to varying successes. However, each of these approaches has their pros and cons. For example, combination medication therapies have the advantage of (typically) being readily available, FDA-approved medications that healthcare professionals can prescribe to patients. Moreover, this approach may allow for the "fine-tuning" of the proportions of each drug in the

mixture towards an effort of maximizing desirable effects and minimizing undesirable effects. However, fixed-proportion combination opioid medications require overlapping pharmacokinetic characteristics of each drug, such as onset and duration of action. On the other hand, bivalent ligands have the capacity to bypass the requirement for pharmacokinetic concordance, as a single molecule will activate both receptors of interest. However, these bivalent compounds would require a longer time to develop and move through the drug development stages to ensure safety and efficacy compared to existing and approved medications. Moreover, this approach may only be viable for certain receptor systems with similar enough binding pockets to allow an overlap in the molecule's pharmacophore composition such that the molecule could appreciably activate both receptors. It seems likely that the best choices between these two approaches would be on a case-by-case basis, but experiments directly comparing the two could greatly elucidate the situations under which each approach would be the most beneficial

4. Fixed-proportion opioid agonist and antagonist interactions

The main finding of Chapter 4 was that fixed-proportion mixtures of a competitive agonist and antagonist at a common receptor produced antagonist proportion-dependent decreases in efficacy. These results showed the usefulness of agonist-antagonist fixed-proportion combinations to predict a "window of effect" detectable by various assays. In this case, the windows of effect of MOR agonists for warm water tail withdrawal at both 50 and 54 °C in nonhuman primates was determined. This could allow for researchers to probe a wide variety of different assays and compare the efficacy requirements for agonists active at various receptor systems to produce an effect in a given assay. Recently, this approach has been shown to be

reproducible with rats in both a tail-withdrawal and drug-discrimination procedure utilizing MOR agonists (Schwienteck, 2019).

Efficacy is defined as a ligand's ability to activate a receptor to generate a response in a biological system and has long been known to be a major determinant in drug effects both in vivo and in vitro (Blumenthal and Garrison, 2011). One of the major themes of this dissertation was determining the role of MOR efficacy in production of behavioral effects (e.g. antinociception and decreases in operant rates of responding) alone and in combination with other compounds (i.e. NMDA antagonists, NOP agonists, and naltrexone). This work further expanded upon the concept of efficacy requirements for the detection of a biological effect in in vivo behavioral assays such that moderate efficacy MOR agonists (e.g. buprenorphine) are incapable of producing (1) meaningful antinociception at higher intensity noxious stimuli (ie. 54°C) and (2) significant decreases in operant rates of responding, both of which high efficacy agonists (e.g. fentanyl) are capable of producing.

These reported results support previous suggestions that efficacy requirements to produce antinociception and other effects can be ranked, and the current data would propose the efficacy-effect ranking of these procedures as 50°C antinociception < schedule-controlled responding < 54°C antinociception (Walker et al, 1993; Banks et al, 2010; Maguire and France, 2014). These findings suggest interesting implications for both preclinical research and clinical practice as they propose the potential for behavioral assays to be ranked based upon their sensitivity for detection of biological effects along a specific range of drug efficacies. If the receptor system has been adequately probed, efficacy-effect procedures could be a useful tool to inform researchers of the proper assay to employ when investigating a relatively novel compound to ensure detection of any potential biological effects. One limiting factor, however, is that it is unknown if

these results would be supported with drugs active at a system other than the MOR. One would hypothesize that the results are translatable due to the generalizability of the properties of "efficacy" across a range of drug classes. Clinically speaking, these efficacy-dependent effects support the idea that low efficacy MOR agonists are effective in reduction of more moderate pain states with a lower incidence of undesirable side effects (Davis, 2012), while higher efficacy MOR agonists would be more effective in treatment of more severe pain states. Clinically, this could provide a tool for physicians to tailor drug effects to the need of the patient. For example, this approach could be used to combine fentanyl and naltrexone at a precise fixed-proportion to mitigate a patient's moderate pain symptoms without the potential for producing respiratory depressant effects, such as with buprenorphine, but permitting a higher degree of antinociceptive efficacy.

5. In vivo investigation of NAQ in rhesus monkeys

This work was the first evaluation of the in vivo efficacy of the novel MOR ligand, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6α-(isoquinoline-3-carboxamido)morphinan (NAQ) in monkeys. NAQ was shown to be ineffective in producing any suppression of operant rates of responding in the assay of schedule-controlled responding and was shown to be ineffective in producing antinociception in the tail withdrawal assay. These effects were consistent up to doses which both (1) antagonized the antinociceptive effects of fentanyl in the tail-withdrawal procedure, and (2) were maximally allowed due to solubility constraints. These results were generally consistent with those in rats, but subtle species differences did emerge. One study reported no significant decreases in rates of responding in a drug discrimination procedure or significant antinociception in a tail-flick procedure (Schwienteck et al, 2019), and

another reported no significant antiallodynia but a significant decrease in rates of food-maintained responding (Siemian et al, 2016). The antagonistic effects of NAQ were also generally consistent with previously reported effects in both mice and rats (Yuan et al, 2015; Siemian et al, 2016; Schwienteck et al, 2019). Overall these results demonstrate a concordance in NAQ activation of the MOR receptor at the organismal level from rodents to nonhuman primates and further support the utility of NAQ as a low efficacy MOR ligand.

Although the modeling resulted in NAQ being quantified as 0.25 proportions of fentanyl, this was still shown to be insufficient to detect any behavioral effect in either of our assays. This suggests that in either schedule-controlled responding or warm-water tail withdrawal, that ligands producing ≤ ~25% of fentanyl's receptor activation will fail to produce a detectable effect suggesting that these procedures are only useful in probing higher efficacy MOR compounds (≥ ~50% of fentanyl receptor activation). In light of this the need to both (1) develop new and (2) identify existing behavioral assays which will allow for the detection of effects along the lower 50% of the fentanyl proportion efficacy range is an imperative goal. One such potential assay to detect effects of lower efficacy MOR agonists could be intracranial self-stimulation. For example, NAQ has been shown to produce detectable behavioral effects including rate-decreasing effects in this procedure in opioid-naïve rats and weak facilitation in opioid-exposed rats (Altarifi et al, 2015; Moerke & Negus, 2019). Moreover, drug discrimination procedures may also provide an avenue for detection of behavioral effects mediated by lower efficacy MOR ligands as NAQ was reported to occasion fentanyl-appropriate responding in rats trained to discriminate fentanyl (Schwienteck et al, 2019). Overall, the literature supports the utility of NAQ as a low efficacy MOR ligand in preclinical pharmacological research.

6. Experimental design considerations for future preclinical pain research

Tail-Withdrawal Procedure

The warm-water tail withdrawal procedure has been historically used in the field of behavioral pharmacology to evaluate the antinociceptive effects of novel compounds, including novel MOR agonists. However, the warm-water tail withdrawal procedure is not without limitations and the predictive validity of non-opioid candidate analgesics in the warm-water tail withdrawal procedure has not been great. One limitation is that the procedure relies entirely upon a reflexive behavior that produces solely a transient nociceptive response that can be "escaped" by removal of the tail from the noxious stimulus (e.g. heated thermos) (Negus, 2019). Because the tail withdrawal behavior relies on a reflex arc within one area of the spinal cord (Weng and Schouenborg, 1996), the receptors mediating any antinociceptive effect may be (1) fewer in number than those responsible for more complex pain states and (2) limited to sub-cortical areas which are crucial in the emotional aspect of pain perception (Negus, 2019). In support of this hypothesis, activation of peripheral opioid receptors is sufficient to produce antiallodynia in tail-withdrawal procedures (Ko et al, 1998, 1999, 2002), however neither peripheral nor centrally activating KOR ligands are currently clinically utilized analgesics. This result suggests a major limitation in the tail-withdrawal procedure if our intention is to translate results to a clinical situation.

The tail-withdrawal procedure does appear to model aspects of acute pain; for example, some of the most robustly effective compounds to produce antinociception in this procedure are MOR agonists and MOR agonists are still the most commonly utilized clinical interventions for moderate to severe acute pain (e.g. post-operative pain states) (Whiteside et al, 2008). However, the translatability of the tail withdrawal procedure to human pain states is highly problematic for

three major reasons: (1) the high potential for false-positives, (2) the issue of dose-requirements to detect some effects, and (3) the inability to detect effects for clinically utilized analgesics (e.g. NSAIDs).

Because the tail withdrawal procedure is a pain-stimulated endpoint (meaning that the measured response increases in duration, intensity, or frequency in the presence of the noxious stimulus), it is highly susceptible to false-positives from compounds that produce motor impairment (Negus, 2019). If a drug is capable of decreasing behavior, then the drug should decrease the tail withdrawal response (indicative of antinociception in this procedure) at some dose. This phenomenon is well characterized; for example, MK-801, PCP, THC, loperamide, and nalfurafine have all been reported to produce decreases in tail withdrawal behaviors in this procedure despite none of these compounds being clinically prescribed analgesics (France et al, 1989; Endoh et al, 2001; Butelman et al, 2004; Maguire and France, 2014). Furthermore, the conflicting reports on the antinociceptive effects of Ro 64-6198 in this procedure obscure the determination of whether a candidate analgesic is a clinically effective analgesic compound (i.e. a compound sufficiently capable of reducing nociceptive signaling rather than simply producing a motor-depressant-related reduction in pain responding) or an example of a false-positive (Ko et al, 2009; Cornelissen et al, 2019).

The second major limitation precluding this procedure from being a strong predictive translation model is the major discrepancies in dose-requirement for detection of an effect. The doses typically required to produce an antinociceptive response in assays utilizing endpoints such as tail-withdrawal tend to be nearly an order of magnitude greater than typically required for more post-operative cases in humans (Whiteside et al, 2008). For example, the dose of methadone (3.2 mg/kg) required in these experiments to produce 100%MPE at 54°C also

produced maximal behavioral suppression in the assay of schedule-controlled responding, and occasionally required antagonist-reversal. This suggests that the efficacy requirements for antinociceptive effects are much greater in a preclinical tail withdrawal procedure than those required for analgesic effects in clinical settings. One negative consequence of these discrepancies could be that the dose-ratios of opioid combinations chosen for preclinical experiments (especially if based upon antinociceptive ED_{50}'s) could be massively greater than those clinically relevant. This could result in determining drug and dose relationships that are entirely irrelevant for translatability and may be entirely different than the relationships between those drugs at clinically relevant dose-ratios. Therefore, not only is this procedure capable of producing false-positives, but potentially also false-negatives.

Finally, this procedure is also severely limited in its ability to produce highly translatable results due to the challenging nature of chronic dosing in such experiments. Pain remains a chronic condition in a number of patients requiring novel and improved treatments. Clinically relevant experimental designs might necessitate more chronic administration to include variables such as antinociceptive tolerance and metabolic alterations, which are likely to greatly impact the progression and treatment of pain in humans (Whiteside et al, 2008).

Schedule-Controlled Responding

Schedule-controlled responding provides one complementary dependent measure to determine behavioral selectivity when using a pain-stimulated behavior such as the warm-water tail withdrawal procedure to study antinociception. In addition, the schedule-controlled responding procedure allowed for the calculation of an experimental "therapeutic index" as one measure of the range of the –fold doses at which a drug can be administered without recruiting a

specific undesirable effect. For example, aim 1 determined the experimental therapeutic index for oxycodone to be five, meaning that oxycodone was five times more potent to produce antiallodynia than sedation. This index does provide some clinical utility and translatability as one of the major goals for pain management is to adequately mitigate pain symptoms and restore daily life function and improve quality of life metrics (Wells et al, 2008). Although this measure is useful to identify selectivity in antinociceptive effects and may have some clinical relevance, it is maybe potentially more fruitful to employ a multi-modal assessment of a number of undesirable side effects in future experiments.

Arguably, the most detrimental undesirable MOR agonist effects of greatest concern are lethality and abuse; these endpoints would be best measured via investigation of respiratory depression and reinforcing effects. In support of these MOR agonist effects being of the greatest concern, and therefore the most important to monitor in any potential opioid-combination experiments, is the Volkow and Collins (2017) report on addressing the opioid crisis which outlined the need for novel opioid reversal interventions, opioid use disorder treatments, and non-addictive treatments for pain. In light of this, there may be side effects of greater significance to investigate in opioid-combination experiments (in an attempt to potentially mitigate) as compared to sedation. This will be further elaborated upon in the proposed alternatives section.

Proposed Alternatives

1) Future preclinical pain research should utilize complementary pain-stimulated and pain-depressed behaviors to assess therapeutic effects of novel analgesics and analgesic adjuncts. Employing both pain-stimulated and pain-depressed behavioral assays would likely mitigate the probability of false-positive analgesic compounds. Moreover, the clinical experience of pain

would be more closely modeled by pain-depressed behaviors, thus would likely serve as a strong predictor of clinical outcomes.

2) Future preclinical pain research should also utilize complementary behaviors to assess undesirable effects. For example, two prominent undesirable effects of MOR agonists are respiratory depression and abuse liability, in addition to behavioral depression/sedation. This is an imperative measure to employ in future research because of the danger that these effects can have on both the user and the society, thus it is important to monitor that adjuncts are not exacerbating the abuse potential or lethality of the opioids they are being combined with.

3) Given the additional ethical considerations involving the use of nonhuman primates in preclinical pain research, the utility of nonhuman primates as research subjects in future preclinical pain research will be limited. Thus, future experiments should aim to utilize rodent models and strive to develop novel paradigms to best improve translatability.

However, if the preclinical approach to opioid combination medication is to continue being utilized, even in a solely informative capacity, it is imperative for this body of data to further inform improvements that can be made. Firstly, there needs to be a paradigm shift in the field moving from the antiquated reliance upon pain-stimulated behaviors to probe antinociceptive effects to employments of pain-depressed behaviors. These behaviors are much more similar to the human clinical condition of pain and are hypothesized to have higher predictive validity of analgesic effectiveness in humans (Negus, 2019). Secondly, complementary measures of undesirable effects should be more focused on investigating the clinically concerning MOR side effects such as respiratory depression and abuse liability. Thus, opioid-combination experiments should be more likely to include these secondary measures probed with assays such as plethysmography, self-administration, or drug-discrimination to

determine the potential of candidate receptor systems to mitigate lethality and abuse cause by MOR agonists. Finally, preclinical experiments should be designed to more closely resemble a human pain condition in which the patient's treatment is impacted by factors such as antinociceptive tolerance and metabolic factors. This would be accomplished with more long-term studies with increased daily dosing frequency to more accurately model the regimen of patients receiving pain medication. Moreover, a less transient and "escapable" pain stimulus would more likely reflect the progression of the patient's state as these factors are very likely to impact the ability of the analgesic to effectively relieve pain.

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