TRUNCATED MU OPIOID RECEPTOR SPLICE VARIANTS AS TARGETS FOR POWERFUL PAIN RELIEF WITH REDUCED SIDE EFFECTS

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Relief from pain is a major goal of all branches of medicine, and poorly controlled pain is associated with substantial economic and human burden. Mu opioid receptors are targets for strong pain relief, mediating the analgesia of widely used analgesic drugs like morphine, hydrocodone, oxycodone, methadone, and fentanyl. Unfortunately, these drugs are associated with numerous side effects such as constipation, respiratory depression, physical dependence, abuse, and addiction.

The mu opioid receptor gene undergoes extensive alternative splicing, although the physiological relevance of many of these splice variants is only now becoming clear. In particular, some of these splice variants produce truncated receptors possessing only 6 transmembrane domains, short of the canonical 7 transmembrane domain structure conserved across the G-protein coupled receptor superfamily. Initial studies suggested that these receptors were expressed in low levels relative to full length variants, and could not bind mu agonist drugs when they were transiently expressed in cell lines, calling into question their importance in opioid receptor pharmacology.

Here, we show that these truncated receptors are in fact targets for a new pain reliever, IBNtxA, which is a potent pain reliever in mice and rats, yet exhibits a dramatically improved side effect profile over current clinically-used mu analgesics. IBNtxA shows only mild constipation, does not depress respiration or produce physical dependence, and shows neither rewarding nor aversive behavior in a conditioned place preference assay. We also examined the pharmacology and regional distribution of this target in rat brain using a radioiodinated form of the drug. Photoaffinity labels based on

the structure of IBNtxA were also synthesized and characterized, and we present evidence consistent with the labeling of truncated mu opioid receptor splice variants in mouse brain.

Other pain relievers were also found to require these truncated receptors for their analgesic effects, such as the clinically used opioid buprenorphine, as well as $kappa_1$ agonists and clinically used $alpha_2$ agonists. Importantly, dose- or use-limiting side effects for each of these drugs were found to be present in knockout animals lacking the truncated splice variant isoforms, suggesting that the desired analgesic properties can be separated from their unwanted side effects.

BIOGRAPHICAL SKETCH

Steven Grinnell graduated from Hampshire College in 2007 with a concentration in Neuroscience, writing an undergraduate thesis entitled “CB1 and Mu Opioid Receptor Oligomerization: A Literature and FRET Study” under advisors Dr. Christopher Jarvis and Dr. Cynthia Gill. After his undergraduate studies, he worked as a post-baccalaureate IRTA fellow at the National Institute of Drug Abuse in the laboratory of Dr. Roy Wise, in collaboration with Dr. Greg Elmer of the University of Maryland at Baltimore at the Maryland Psychiatric Research Center.

In 2008, Dr. Grinnell joined the doctoral program in Neuroscience at the Weill Cornell Graduate School of Medical Sciences where did his thesis research under Dr. Gavril Pasternak, Anne Burnett Tandy Chair of Neurology at Memorial Sloan Kettering Cancer Center.

Dr. Grinnell received a Predoctoral Fellowship in Pharmacology from the PhRMA Foundation in 2012, and has received travel awards to present work at the 2013 and 2014 Experimental Biology conferences from the American Society for Pharmacology and Experimental Therapeutics. He was also invited to present work at the 2012 NIDA/INSERM Workshop on US-France collaboration on Drug Abuse and Addiction Research.

To Parker and Saoirse,

I love your questions.

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Chapter 1: Introduction

"*No one rejects, dislikes, or avoids pleasure itself, because it is pleasure, but because those who do not know how to pursue pleasure rationally encounter consequences that are extremely painful. Nor again is there anyone who loves or pursues or desires to obtain pain of itself, because it is pain, but because occasionally circumstances occur in which toil and pain can procure him some great pleasure."*

Cicero*, On the Ends of Good and Evil*, 45 BC

Pain is an evolutionarily essential behavior. Nociceptive signals maximize the fitness of the individual, disincentivizing actions that result in physical harm and thus endanger the individual's chances of reproductive success. In humans, congenital insensitivity to pain is associated with substantially increased risk of self-injury and subsequent infection, frequently resulting in mortality in childhood (Nagasako et al., 2003). However, in a situation of imminent danger, such as an encounter with a predator, near-term survival outweighs the long-term fitness value of avoiding tissue damage. Thus, the endogenous opioid system appears to have evolved to silence painful stimuli, facilitating "fight or flight" via fear-induced hypoalgesic response (Fanselow, 1986).

It is not surprising, then, that the amelioration of pain should be physiologically associated with reward. Indeed, opiates were likely among the first euphoriant drugs: *Papaver somniferum* was cultivated in Mesopotamia around 3400 BCE in what is now Iraq (Brownstein, 1993). Ancient Sumerians harvested opium from these seed pods, which they called *hul gil* or "the plant of joy." Although the many medical uses of opium were also appreciated many thousands of years ago in the earliest known medical text, the Ebers Papyrus (ca. 1550 BCE), the pharmaceutical and rewarding aspects of the drug have always existed side by side.

As powerful pain relievers, opioid drugs such as morphine, fentanyl, and oxycodone are widely used clinically. Due to their long history and proven efficacy,

these mu opioids are the mainstay in the management of moderate to severe acute and chronic pain, but many patients report inadequate pain control (Moskovitz et al., 2011). In many, side effects such as constipation, nausea and vomiting, sedation, and potential for abuse result in suboptimal dosing or even discontinuation of treatment, and millions of patients suffer poorly controlled pain at substantial human and economic cost estimated at hundreds of billions of dollars annually (Institute of Medicine, 2011). Not surprisingly, major depression, post-traumatic stress disorder, and anxiety disorders are significantly more prevalent in patients with chronic pain according to the National Comorbidity Study (McWilliams, Cox, & Enns, 2003). Furthermore, patients with chronic pain frequently report suicidal ideation (Racine, Choinière, & Nielson, 2014) and are twice as likely as patients without chronic pain to commit suicide (Tang & Crane, 2006).

Clearly, then, there is a significant unmet need for strong analgesic drugs with improved side effect profiles (Moskovitz et al., 2011). Thousands of synthetic opioid compounds have failed to supplant morphine as the "gold standard" for the treatment of severe pain, although studies have failed to show its superiority over other mu opioids (Gálvez & Pérez, 2012). However, decades of research have repeatedly demonstrated the heterogeneity of opioid receptor targets. Further elucidation of this concept of receptor multiplicity will facilitate the development of a new generation of opioid drugs that offer the benefits of powerful pain relief with fewer adverse effects.

Opioid Receptors

The history of opioid receptor pharmacology is inextricably tied to the history of the radioligand binding assay. Although physiologists such as Langley, Hill, and Ehrlich conceived of "receptors" which bound and mediated the actions of drugs in the early 1900s (Rang, 2006), no such neurotransmitter receptor had ever been concretely demonstrated until 1970. Changeux et al (Changeux, Kasai, & Lee, 1970) used a tritium-

labeled acetylcholine receptor ligand, decamethonium, to probe protein extracted from the receptor-rich electric organ of the Torpedo ray. Previously, it had been impossible to differentiate the acetylcholine receptor binding from the enzyme acetylcholinesterase (AChE) binding as the available radioligands exhibited high affinity for both proteins. Changeux used a recently-isolated component of snake venom called alpha-bungarotoxin which bound acetylcholine receptors but not AChE, allowing him to definitively identify specific receptor binding versus nonspecific interaction of the radioligand with other macromolecules such as enzymes.

Still, the discovery that acetylcholine receptors made up nearly 20% of the weight of the peculiar Torpedo ray electric organ led many researchers to doubt that neurotransmitter receptors, expressed at dramatically lower abundance in the brain, would be discovered in their lifetimes (Snyder, 2009). Pioneering pharmacologist Avram Goldstein attempted to use the opiate levorphanol and its inactive enantiomer to demonstrate the existence of a stereoselective receptor for opiates, but was limited by low specific activity of the tritiated levorphanol radioligand (Goldstein, Lowney, & Pal, 1971). However, Goldstein's idea that the criteria of stereoselectivity could be used to determine that observed binding corresponded to the receptor mediating a drug's physiological effects would later prove crucial.

Outside of the CNS, Cuatrecasas had been able to successfully use ^{125}I-labeled insulin to discover the insulin receptor in fat cells (Cuatrecasas, 1971), and Lefkowitz discovered ^{125}I-labeled ACTH binding in the adrenal gland (Lefkowitz, Roth, Pricer, & Pastan, 1970). Finally in 1973, the labs of Sol Snyder andd Lars Terenius, and shortly thereafter Eric Simon used tritiated naloxone, dihydromorphine, or etorphine, respectively, to demonstrate the existence in the mammalian brain of an opiate receptor (C. B. Pert & Snyder, 1973; Simon, Hiller, & Edelman, 1973; Terenius, 1973).

Importantly, while the biologically active (-)-enantiomers of methadone, levallorphan, and levorphanol competed this binding with very high affinity, the inactive (+)-enantiomers exhibited dramatically lower affinity for the site, confirming its identity as the target mediating the physiological effects of opioid drugs.

Opioid Receptor Multiplicity

The first demonstrations of a receptor in the central nervous system for opiates naturally inspired far more questions than they answered. What were brain's endogenous opiate ligands? Where were opiate receptors expressed and how did they mediate their effects? Finally, were there other opiate receptors?

Earlier, clinical studies revealed that low doses of nalorphine antagonized morphine analgesia, but higher doses analgesia returned, which was most easily explained by the existence of multiple opioid receptors (Houde & Wallenstein, 1956; Lasagna & Beecher, 1954). Portoghese had further speculated that as nalorphine was only able to reverse some of the effects of certain Diels-Alder adducts[1] of thebaine (Bentley et al., 1965), "it seems likely that there may be several different species of narcotic analgesic receptors" (Portoghese, 1965, p609).

Shortly thereafter, Martin proposed the existence of "M" and "N" receptors (for morphine and nalorphine) to explain the differences between the effects of various opioid drugs, a concept he called "receptor dualism" (Martin, 1967). After the demonstration of opioid receptors in 1973, Martin et al categorically investigated the effects of different opioid compounds such as morphine, buprenorphine, n-allyl-normetazocine (SKF-10,047), and ketocyclazocine, in the chronic spinal dog (Gilbert &

[1] These resulting *endo*-theno derivatives, known as orvinols and thevinols, include the extremely potent agonist etorphine, mu partial-agonist/kappa antagonist buprenorphine, and mu/delta/kappa antagonist diprenorphine, all of which exhibit very high affinity for opioid receptors but little or no selectivity for any one class of receptor.

Martin, 1976; Martin, Eades, Thompson, Huppler, & Gilbert, 1976). Importantly, they discovered that ketocyclazocine, despite behaving as an analgesic and suppressing the flexor reflex as well as constricting pupils like morphine, did not alter body temperature and only weakly affected pulse and respiratory rate. Furthermore, ketocyclazocine neither strongly precipitated nor ameliorated a withdrawal syndrome in morphine-tolerant animals. On the basis of these studies, Martin proposed the existence of at least 3 opioid receptors, and named them mu (for morphine), kappa (for ketocyclazocine), and sigma (for SKF-10,047). Indeed, we now know that the Bentley compound to which Portoghese was referring, M320, is a full kappa agonist like ketocyclazocine, explaining why its analgesia could not be reversed by the mu antagonist/kappa agonist nalorphine (Lewis, 1999). We also now know that sigma receptors are not in fact opioid at all, although they modulate the effects of opioid analgesia by altering their interactions with G-proteins (Kim et al., 2010).

Hans Kosterlitz had previously championed[2] the use of isolated tissue preparations such as guinea pig ileum and mouse vas deferens to measure the potency and efficacy of opioids *in vitro* ((Gyang, Kosterlitz, & Lees, 1964; Henderson, Hughes, & Kosterlitz, 1972; Kosterlitz & Robinson, 1955, 1957, 1958). His young protégé, John Hughes, had been the first to sequence the endogenous opioid peptides leu- and met-enkephalin (Hughes and Kosterlitz, 1975), his success in no small part due to these *in vitro* assays. Together, the laboratory of Hughes and Kosterlitz would also be the first to demonstrate the existence of multiple opioid receptors *in vitro*, showing that guinea pig ileum primarily contained mu-like receptors, while the mouse vas deferens contained

[2] Notably, the first demonstration of the effect of morphine on the peristaltic action of the isolated guinea pig ileum long predated Kosterlitz. Pioneering German pharmacologist Paul Trendelenburg first reported the qualitative effects of various drugs on this reflex in a seminal 1917 paper. Paton (1956), alongside Kosterlitz and Schaumann (1955), developed Trendelenburg's findings into an effective assay which would permit quantitative *in vitro* comparisons between new opioid derivatives on the basis of their potency and efficacy (Gyang, Kosterlitz, and Lees 1964; recounted in Lees 1998).

another population, named delta (for vas deferens) which differed in its selectivity from Martin's kappa receptor (Lord, Waterfield, Hughes, & Kosterlitz, 1977).

Opioid Receptor Multiplicity: Mu Subtypes

The discovery of multiple opioid receptors failed at first to achieve the holy grail of opiate research – that is, the discovery of a powerful pain reliever lacking the side effects of morphine such as constipation, respiratory depression, and abuse potential. Although full agonists of kappa receptors such as nalorphine and ketocyclazocine were tested in humans as analgesics, they produced psychotomimesis and dysphoria in patients and their use was not further pursued for this indication (Cahal, 1957; Kumor, Haertzen, Johnson, Kocher, & Jasinski, 1986). Lower efficacy agonists at kappa receptors such as pentazocine, a mixed kappa agonist/mu antagonist, were approved with the caveat that higher doses could cause these undesirable side effects; they continue to be used today, albeit rarely, with prescriptions vastly exceeded by those of mu opioid agonists such as morphine, hydrocodone, oxycodone, and fentanyl.

Importantly, although drugs could be grouped into mu, delta, kappa or sigma families, in was clear that not all mu agonists were interchangeable. Clinically, physicians have long appreciated the differences between mu analgesic drugs, within and between patients, and tailored therapy accordingly to minimize side effects such as nausea seen with one drug but not with another (G. W. Pasternak & Pan, 2013). Furthermore, the phenomenon of incomplete cross-tolerance is observed clinically between mu analgesics, permitting the practice of "opioid rotation," whereby a patient highly tolerant to one drug may be switched to another at a lower dose than would be predicted based solely on potency ratios (Cherny et al., 2001). By lowering the dose required to control a patient's pain, opioid rotation often achieves a reduction in the side effects accompanying the higher doses of the patients' former opioid. Clearly, these

clinical observations support a more complex model than single, homogeneous population of receptors mediating mu opioids' analgesic effects.

The first experimental evidence in favor of multiple "mu" subtypes came from investigations with the naloxone-derivative naloxazone. Naloxazone inhibition of binding was found to be very long lasting and resistant to repeated washes - binding was functionally irreversible, unlike the parent compound which behaved as a traditional reversible competitive antagonist (G. W. Pasternak & Hahn, 1980). Consistent with *in vitro* findings, pretreatment of mice with a single dose of naloxazone dramatically shifted the ED_{50} of morphine 11-fold a full day later, unlike naloxone (G. W. Pasternak, Childers, & Snyder, 1980). Surprisingly, despite this loss of analgesia, there was no significant difference in the LD_{50} of morphine, suggesting that the binding of naloxazone to the receptors mediating respiratory depression was rapidly reversible, whereas the blockade of the sites mediating analgesia was very long lived. Naloxone administered to naloxazone-pretreated animals immediately before a lethal dose of morphine prevented death, confirming the opioid nature of the effect. The brains of naloxazone-pretreated animals exhibited wash-resistant loss of a high affinity site (0.8 nM) comprising about 10% of total [^{3}H]-naloxone binding, while the remaining lower affinity binding (2.3nM) was unaffected.

A short time later, the observed effects of naloxazone were found to be mediated in earnest by naloxonazine, a stable dimer of the parent azone compound spontaneously formed under acidic conditions (Hahn, Carroll-Buatti, & Pasternak, 1982; Hahn & Pasternak, 1982). Naloxonazine pretreatment perfectly recapitulated the results obtained with naloxazone, but at doses 20-fold lower (Ling, Simantov, Clark, & Pasternak, 1986). The high affinity binding site associated with morphine analgesia was

named the mu_1 subtype, while the moderate affinity site associated with respiratory depression was named mu_2.

Further studies on mu subtypes revealed that naloxonazine could dissociate morphine analgesia not only from respiratory depression (Ling, Spiegel, Lockhart, & Pasternak, 1985; Ling, Spiegel, Nishimura, & Pasternak, 1983), but also physical dependence (Ling, MacLeod, Lee, Lockhart, & Pasternak, 1984). Despite loss of analgesia in naloxonazine pre-treated rats, animals continuously infused with I.V. morphine continued to express profound withdrawal signs when given a challenge dose of naloxone, suggesting that morphine analgesia and physical dependence were not mediated by the same population of receptors.

These studies also revealed a difference in receptor distribution; both mu_1 and mu_2 sites were found supraspinally, while only mu_2 sites were present in the spinal cord (G. W. Pasternak et al., 1983; Paul, Bodnar, Gistrak, & Pasternak, 1989). Further studies identified mu_2 sites in the gut which mediate the peripheral component of morphine's constipating effects (Gintzler & Pasternak, 1983; Heyman, Williams, Burks, Mosberg, & Porreca, 1988; Paul & Pasternak, 1988). Finally, the spontaneously-occurring mu-deficient CXBK mouse strain was found to have no difference from wildtype animals in the analgesic potency of intrathecal morphine, but a >20-fold shift in i.c.v. morphine (Figure 1; Pick, Nejat, & Pasternak, 1993). The authors further observed that morphine given i.c.v. potentiated a fixed dose of morphine given intrathecally, a synergy that was poorly blocked by the mu_1 antagonist naloxonazine at doses that readily blocked i.c.v. morphine alone – implicating a mu_2-dependent mechanism for supraspinal/spinal synergy. Consistent with these results, CXBK animals exhibited nearly identical supraspinal/spinal synergy to wildtype controls, despite profound loss of analgesia to i.c.v. morphine alone.

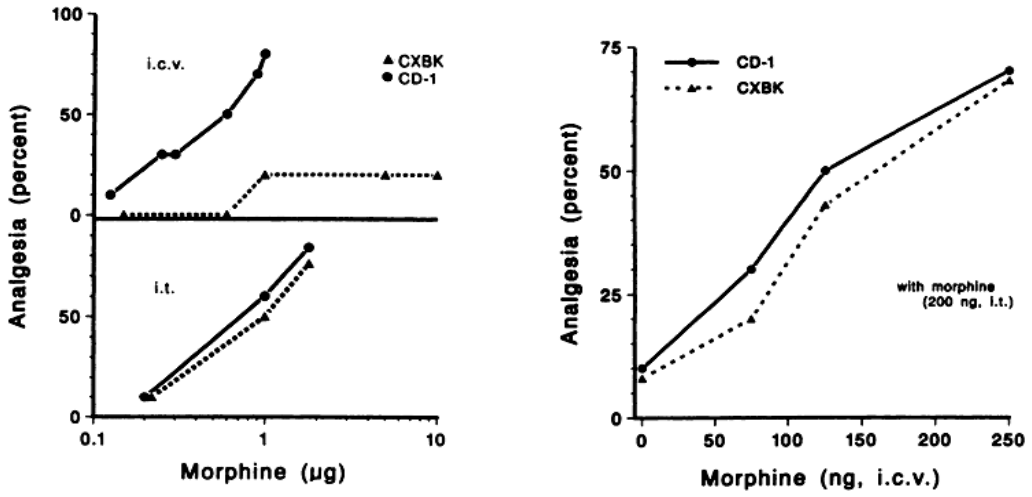


Figure 1: Effect of morphine on CD-1 and CXBK mice.
(Left) i.c.v morphine analgesia is lost while i.t. morphine is intact in the spontaneously mu-deficient CXBK mice, suggesting distinct receptor mechanisms for spinal and supraspinal morphine analgesia. (Right) Despite near-total loss of supraspinal morphine analgesia, there is no difference in the ability of i.t. morphine to potentiate i.c.v. morphine analgesia relative to wildtype CD-1 controls. Reproduced from (Pick et al., 1993).

Opioid Receptor Multiplicity: Kappa Subtypes

Like mu receptors, kappa receptors could also be subdivided based on receptor binding. For many decades, benzomorphans such as ^{3}H-ethylketocyclazocine (a derivative of the prototypical agonist, ketocyclazocine) and ^{3}H-bremazocine were used to study kappa receptor binding, despite the fact that they were not very selective for kappa receptors. Studies looking at kappa binding therefore used tissues which were enriched for kappa receptors and deficient in mu and delta receptors, such as the guinea pig cerebellum and lumbo-sacral spinal cord, or used mu and delta selective blockers to artificially enrich for the desired kappa sites. In the guinea pig lumbo-sacral spinal cord, ^{3}H-bremazocine labeled both classical kappa sites as well as “benzomorphan

sites," termed kappa$_2$ (Attali, Gouardères, Mazarguil, Audigier, & Cros, 1982a, 1982b). These sites could also be observed in guinea pig striatum using ^{3}H-etorphine in the presence of mu- and delta-selective blockers, further supporting a distinction between subtypes (Audigier, Attali, Mazarguil, & Cros, 1982; Gouarderes, Attali, Audigier, & Cros, 1983). The synthesis of the non-benzomorphan kappa-selective small molecule U50,488 (Szmuszkovicz & Von Voigtlander, 1982; Vonvoigtlander, Lahti, & Ludens, 1983) and subsequent resolution of its enantiomers (DeCosta, George, Rothman, Jacobson, & Rice, 1987) dramatically simplified the differentiation between kappa sites, as kappa$_1$ binding was U50,488-sensitive while kappa$_2$ binding was U50,488-insensitive (Rothman et al., 1989; Zukin, Eghbali, Olive, Unterwald, & Tempel, 1988). Still, the whether all of these studies were measuring the same kappa sites is unclear given the different tissues and radioligands used to define the binding.

Competition studies further fragmented kappa binding, as Clark et al (1989) demonstrated that even kappa$_1$ sites were not a homogeneous population. Despite monophasic competitions by kappa ligands such as U50,488, tifluadom, Mr2034, and the endogenous kappa peptide Dynorphin A (1-17), both ^{3}H-ethylketocyclazocine and ^{3}H-U69,593 binding in guinea pig cerebellum was clearly biphasic when competed by the endogenous peptides Dynorphin B and alpha-neoendorphin, leading the authors to declare the existence of kappa$_{1a}$ and kappa$_{1b}$ sites (Clark et al., 1989). In the same paper, Clark et al demonstrated yet another site, named Kappa$_3$, which was bound reversibly by a new naloxone derivative, ^{3}H-naloxone benzoylhydrazone (NalBzoH). The classification of this site as a kappa receptor subtype was justified by high affinity for the prototypical kappa ligand, ketocyclazocine, as well as a number of other kappa drugs such as Mr2034 and WIN44,441, yet it was insensitive to the kappa$_1$-selective agonist U50,488 and antagonist norBNI. Binding also clearly differed from the previously

described $kappa_2$ sites as $kappa_3$ binding was competed with high affinity by ethylketocyclazocine and tifluadom.

Behavioral studies supported the preliminary characterization of a separate $kappa_3$ site labeled by NalBzoH. Although its binding was nonselective, at low doses it antagonized both mu_1 and mu_2 analgesia independently of one another, which was shown by blocking i.c.v. or i.t. DAMGO analgesia (Gistrak, Paul, Hahn, & Pasternak, 1989). Consistent with its characterization as a mu antagonist, NalBzoH also prevented morphine lethality and constipation. It also antagonized delta analgesia produced by i.t. DPDPE as well as $kappa_1$ analgesia produced by s.c. U50,488. As with nalorphine years earlier, though, at higher doses, NalBzoH was itself analgesic, and animals were not cross-tolerant with either morphine or U50,488 – reinforcing the conclusion that $kappa_3$ receptors could be an important target for powerful pain relief with mitigated side effects.

Evidence for a distinct $kappa_3$ receptor was bolstered further by re-examination of the behavioral pharmacology of mixed agonist-antagonist drugs nalorphine and nalbuphine which had been around for decades. Indeed, nalorphine's differing actions in the chronic spinal dog had been the original basis for Martin's proposal of a distinct "N" opioid receptor in addition to an "M" receptor that mediated the effects of morphine (Martin, 1967). Nalorphine had since been classified as a mu antagonist/kappa agonist, yet new studies found its analgesia to be insensitive to the $kappa_1$ antagonist norBNI (Paul, Pick, Tive, & Pasternak, 1991). Furthermore, animals made tolerant to nalorphine were not cross-tolerant to morphine or U50,488, while they were cross-tolerant with naloxone benzoylhydrazone. Finally, animals made tolerant to U50,488 were not cross-tolerant with nalorphine – strong evidence that the

target mediating its analgesia was not the kappa$_1$ receptor but rather the newly discovered kappa$_3$ receptor.

Nalbuphine, another drug classified as a mu antagonist/kappa agonist, was also found to interact with kappa$_3$ receptors supraspinally *in vivo*, although analgesia was readily reversed reversed by i.t. norBNI, consistent with kappa$_1$-dependent analgesia at the spinal level (Pick, Paul, & Pasternak, 1992). U50,488 and nalbuphine analgesia was synergistic, rather than additive, supporting this interpretation. Finally crosstolerance between nalbuphine and U50,488 was unidirectional, with U50,488-tolerant animals showing tolerance to nalbuphine, but not the reverse; however, animals were bidirectionally crosstolerant to the kappa$_3$ agonists NalBzoH and nalorphine, reinforcing the partial role for kappa$_3$ receptors in nalbuphine's actions.

Cloning of the Opioid Receptors

The advent of molecular biology techniques ushered in a new era of opioid pharmacology. Kieffer et al and Evans et al independently cloned the first (delta) opioid receptor by screening cDNA libraries created from NG108-15 cells (Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992). This cell line, produced by fusion of mouse NG18TG2 neuroblastoma and rat C6 glioma cells, natively expresses delta opioid receptors and had been used to great effect as the first *in vitro* preparation of opioid receptors derived from cell culture (Klee & Nirenberg, 1974), leading to the discoveries that opioid receptors are negatively coupled to adenylate cyclase activity (Sharma, Nirenberg, & Klee, 1975) and that adaptations in cAMP regulation in response to chronic opioid treatment could explain phenomena such as tolerance and dependence (Sharma, Klee, & Nirenberg, 1975). These hybrid cells would further prove essential in establishing the actions of the delta opioid receptor as guanosine nucleotide-dependent (Koski & Klee, 1981; Sharma, Klee, & Nirenberg, 1977)

and mediated by a pertussis-toxin sensitive member of the G-protein family (Burns, Hewlett, Moss, & Vaughan, 1983; Hsia, Moss, Hewlett, & Vaughan, 1984; Kurose, Katada, Amano, & Ui, 1983).

After transfection of COS cells with fractions of the cDNA library, Kieffer et al used binding by the delta-selective enkephalin derivative [^{3}H]-DTLET to screen for positive pools of cells which were then fractionated recursively until a single clone was isolated. Evans et al transfected COS cells with the entire library, then screened colonies by autoradiography with another enkephalin derivative, [^{125}I]-DADLE – which, although not selective for delta receptors owing to its affinity for mu_1 sites (Nishimura, Recht, & Pasternak, 1984), had the benefit of dramatically higher specific activity of ^{125}I rather than ^{3}H. Unsurprisingly, both Kieffer and Evans discovered the same cDNA which conferred delta peptide binding activity to transfected cell membranes with a selectivity profile consistent with delta sites.

Shortly after this discovery, Chen et al used the mouse DOR-1 cDNA to probe a rat cDNA library with low hybridization stringency; the researchers isolated a cDNA which conferred on COS cells [3H]-diprenorphine binding competed with nanomolar affinity by the highly mu-selective enkephalin derivative, DAMGO (Chen, Mestek, Liu, Hurley, & Yu, 1993). Binding was also competed with high affinity by mu-selective small molecules such as naloxonazine, b-funaltrexamine, and cyprodime, while the delta-selective peptides DPDPE and DSLET and kappa-selective small molecule U50,488 had dramatically lower affinity. Finally, the authors demonstrated that the receptor was negatively coupled to adenylate cyclase, confirming that it was a member of the G-protein coupled receptor family as expected of the true mu opioid receptor. A number of other labs independently reported sequences of the mu receptor in the ensuing

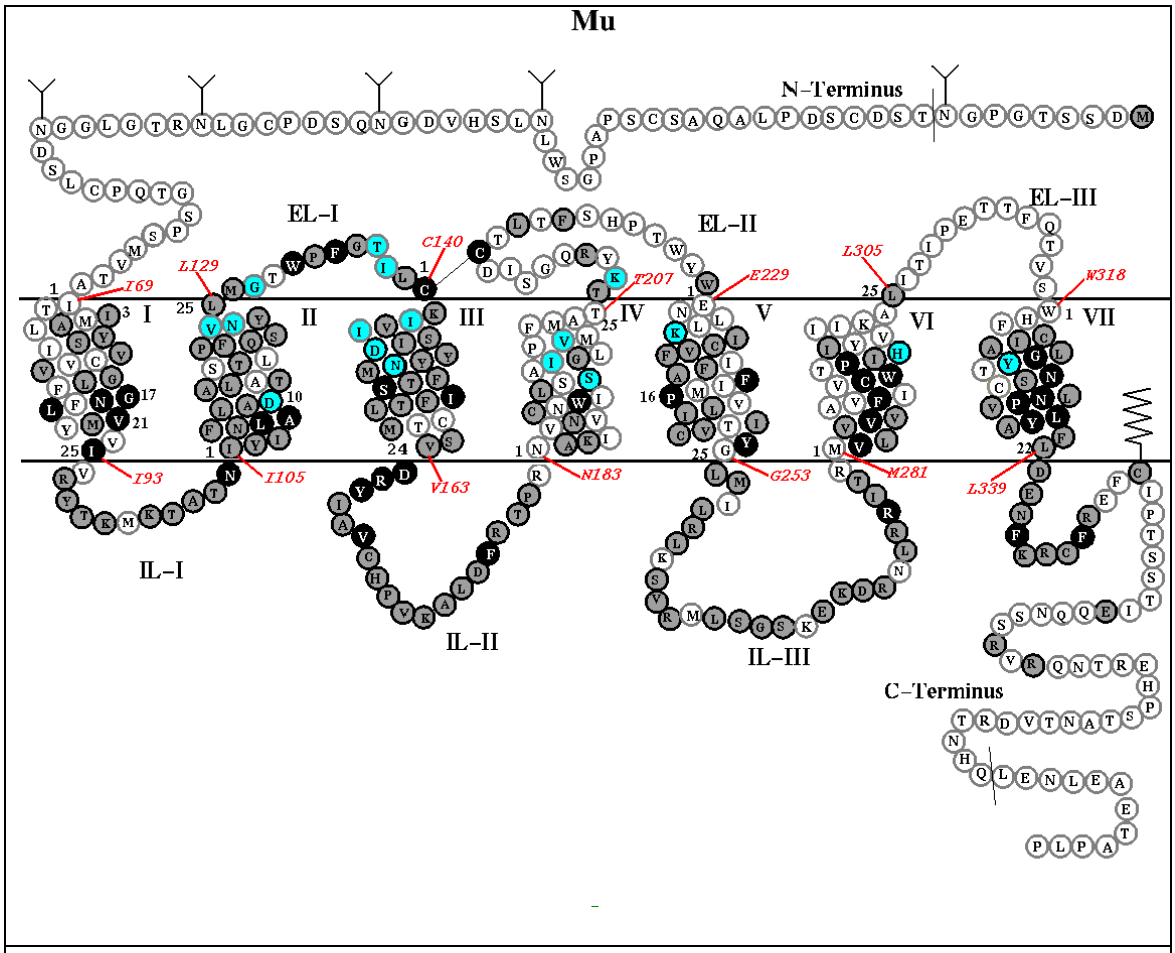


Figure 2: Serpentine model of the rat MOR-1 amino acid sequence and opioid family homology.
Residues are denoted by their 1-letter code, with Roman numerals denoting transmembrane domains and intra/extracellular loops. Residues highly conserved across the rhodopsin-like class A GPCR subfamily are shown in black, while residues conserved across mu, delta, and kappa receptors are shown in gray. Reproduced from (Subramanian, Paterlini, Portoghese, & Ferguson, 2000).

months (Fukuda, Kato, Mori, Nishi, & Takeshima, 1993; R. C. Thompson, Mansour, Akil, & Watson, 1993; Wang et al., 1993).

Kappa opioid receptors were cloned serendipitously by Yasuda et al, who were screening a mouse cDNA library with probes for the somatostatin receptor family (Yasuda et al., 1993). Two clones were isolated with approximately 61% sequence

identity to one another and 35% sequence identity to the somatostatin receptor SSTR1; however, expression of these clones in COS cells did not result in binding of somatostatin peptides but rather opioids, possessing delta- and kappa-like pharmacology respectively. The rat kappa opioid receptor was reported independently just weeks later by Minami et al and in the ensuing months, a number of other labs reported independent discoveries of kappa receptors as well (Chen, Mestek, Liu, & Yu, 1993; S. Li et al., 1993; Meng et al., 1993; Minami et al., 1993).

Overall, the mu, delta, and kappa receptors show about 70% sequence homology in the transmembrane regions and 60% in the loop regions; however, whereas the intracellular loops show about 90% homology, the N- and C- termini and extracellular loops 2 and 3 show virtually none (Figure 2; Kane, Svensson, & Ferguson, 2006). Across rodent and human receptors, there is >90% sequence homology (Knapp et al., 1995). All three receptors are members of the rhodopsin-like class A GPCR sub-family and are negatively coupled to adenylate cyclase via a pertussis toxin sensitive G-protein mechanism (Piros, Hales, & Evans, 1996).

A fourth, previously unknown member of the opioid receptor family was cloned independently by a number of labs, sharing 45-55% similarity overall with the mu, delta, and kappa receptors ((Bunzow et al., 1994; Mollereau et al., 1994; Y-X Pan, Cheng, Xu, & Pasternak, 1994; Wick et al., 1994). Named by Mollereau et al as “Opioid Receptor-Like” or ORL, this receptor is actually evolutionarily more ancient, and provides a bridge from a common ancestor from which all vertebrate opioid receptors descend. ORL and KOR can be grouped together on the basis of their greater similarity, as can MOR and DOR, as is evident from a phylogenetic analysis of their sequences (Figure 3, (Stevens, Brasel, & Mohan, 2007)).

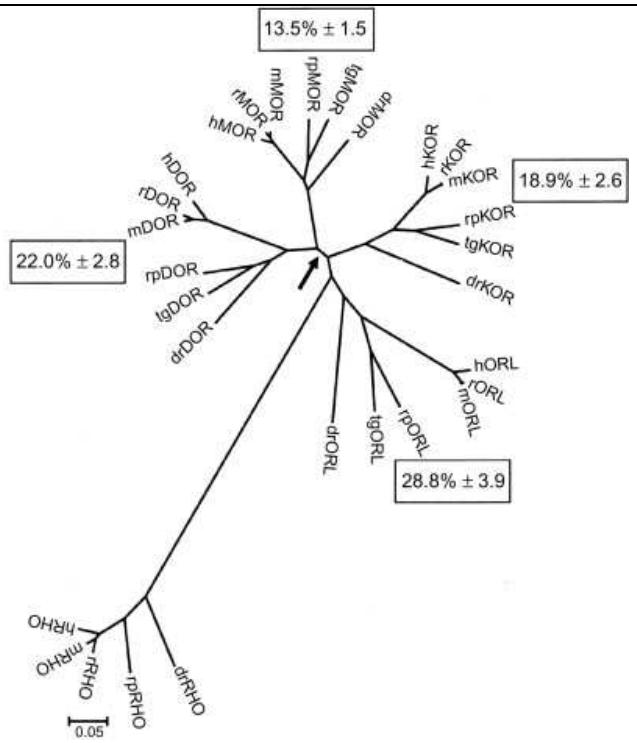


Figure 3: Opioid receptor phylogeny in vertabrates.
Mu (MOR), Delta (DOR), and Kappa (KOR) opioid receptor sequences from six vertebrate species were used to generate a phylogenetic tree, rooted with rhodopsin (RHO). Values in each box represent the mean ± SEM diverengence within each receptor family, and length of each branch is proportional to the differences of each sequence. The arrow denotes the bifurcation between MOR and DOR sequences from KOR and ORL sequences. Human, *Homo sapiens* (h); mouse, *Mus musculus* (m); rat, *Rattus norvegicus* (r); leopard frog, *Rana pipiens* (rp); rough-skinned newt, *Taricha granulosa* (tg); and the zebrafish, *Danio rerio* (dr). Reproduced from (Stevens, 2015).

Mu Opioid Receptor Multiplicity Redux: Antisense, Splicing, and M6G

The discovery of single genes for each of mu, delta, and kappa opioid receptors seemed at first to be at odds with the vast literature on receptor subtypes as defined pharmacologically. However, knowledge of the gene sequences permitted the design of antisense oligonucleotides directed toward specific regions of the receptor mRNA, resulting in the selective degradation of these mRNAs by the endonuclease RNase H or steric blocking of translation ((Dias & Stein, 2002). Thus, the next phase in the story of

receptor multiplicity involved the use of these probes, which exhibited dramatically more selectivity than the available agonist and antagonist drugs previously used to define receptor subtypes.

The Pasternak laboratory utilized antisense oligonucleotides (ODNs) directed against the newly discovered MOR-1 (G. Rossi, Pan, Cheng, & Pasternak, 1994), DOR-1 (Standifer, Chien, Wahlestedt, Brown, & Pasternak, 1994), and KOR-1 (Chien & Pasternak, 1995) genes, confirming that the loss of these single genes alone was sufficient to impair analgesia of morphine, DPDPE, or (-)-pentazocine, respectively. Critically, however, antisense ODNs targeted to different regions of the receptor mRNA did not uniformly block the analgesia produced by mu, delta, or kappa analgesics (K. R. Pasternak, Rossi, Zuckerman, & Pasternak, 1999; G C Rossi, Pan, Brown, & Pasternak, 1995; Grace C Rossi, Su, Leventhal, Su, & Pasternak, 1997), suggesting that while a single gene mediated their effects, the complex subtypes observed pharmacologically might be explained by the existence of splice variants of these receptor genes.

Organisms have evolved metabolic pathways to minimize the toxicity of xenobiotic compounds, acting to increase the water-solubility of the parent compound to facilitate elimination in urine. Whereas phase I biotransformations of xenobiotics consist of oxidation, reduction, and hydrolysis reactions to unmask polar functional groups on the parent molecule, phase II biotransformations involve conjugating polar moieties to the parent molecule – primarily in the form of sulfate, glutathione, and glucuronic acid conjugates (Liston, Markowitz, & DeVane, 2001). Most phase II biotransformations yield metabolites with dramatically decreased activity at their cognate receptors relative to their parent forms, but M6G is an important exception. Although the conjugation of the phenolic 3-OH position of the morphinan scaffield by the UDP-dependent glucuronide transferase enzyme UGT2B7 yields a compound with

negligible affinity for mu receptors, the affinity of M6G for mu receptors is actually somewhat higher than morphine itself (Coffman, Rios, King, & Tephly, 1997; G. W. Pasternak, Bodnar, Clark, & Inturrisi, 1987). Given subcutaneously, M6G is approximately 2-fold more potent than morphine in a tail flick assay. However, given i.c.v. or i.t., M6G is 90- and 650-fold more potent than morphine, respectively, which cannot be explained on the basis of binding affinity or efficacy at MOR-1 alone (Paul, Standifer, Inturrisi, & Pasternak, 1989). The difference between systemic and supraspinal/spinal potencies is believed to be pharmacokinetic, as the polar glucuronide moiety reduces the ability of the drug to cross the blood brain barrier (Yoshimura, Ida, Oguri, & Tsukamoto, 1973).

The dramatically increased potency of M6G has important clinical ramifications – the area under the curve (AUC) in plasma for M6G exceeds morphine by a factor of 9:1 after morphine oral administration (Osborne, Joel, Trew, & Slevin, 1990), and may be substantially higher in patients with impaired renal function (Osborne, Joel, & Slevin, 1986). In fact, after taking into account the relative potencies of morphine and its glucuronide metabolites, concentrations measured in cerebrospinal fluid suggest that a majority of the analgesic effect observed after morphine administration is actually due to M6G rather than morphine itself (Hand et al., 1987). Recent estimates suggest that as much as 96.6% of the analgesic effect of an oral dose and 85.4% of an intravenous dose of morphine is likely due to M6G rather than morphine (Klimas & Mikus, 2014).

Using antisense mapping, Rossi et al demonstrated that antisense ODNs targeted to exons 1 and 4 blocked supraspinal morphine analgesia, while ODN's targeted to exons 2 and 3 failed to have any significant effect (G C Rossi, Pan, et al., 1995). In contrast, supraspinal M6G analgesia was blocked by antisense ODNs targeted to exons 2 and 3 but not exons 1 and 4. The strict exon boundaries observed by antisense mapping

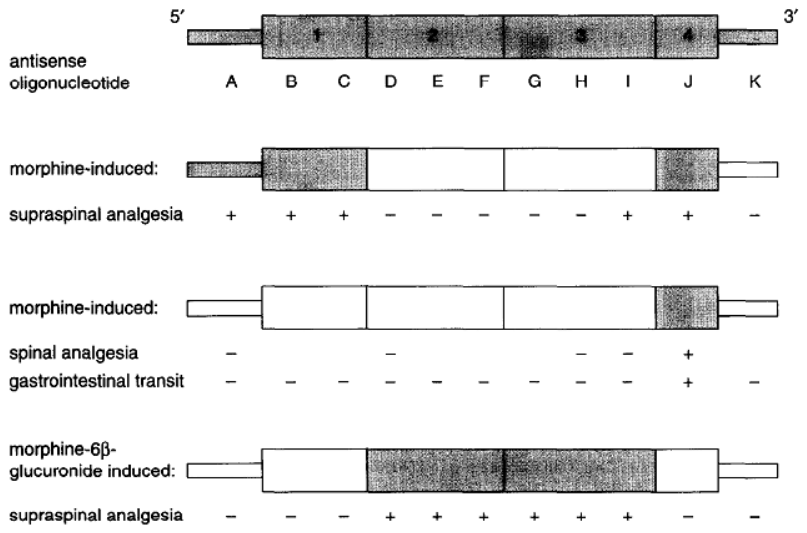


Figure 4: Antisense mapping MOR-1.
Antisense probes designed against different exons of the MOR-1 gene shows differential exon requirements for morphine and M6G analgesia, supporting a model where different splice variants mediate the effects of each drug. Reproduced from (G. W. Pasternak & Standifer, 1995), based on data from (G C Rossi, Pan, et al., 1995).

suggested that alternative splicing could explain the pharmacological differences observed between morphine and M6G despite the presence of only one mu opioid receptor gene.

In rats, antisense ODNs directed against the 5' untranslated region (UTR) encoded by exon 1 of the MOR-1 gene significantly attenuated morphine and DADLE analgesia, but were ineffective against M6G, consistent with the results obtained in mice and reinforcing the importance of exon 1 in morphine but not M6G analgesia (G C Rossi, Standifer, & Pasternak, 1995). Antisense ODNs directed against G-proteins suggested that effector pathways activated by morphine or M6G diverged as well. In rats, $G_i\alpha 2$ probes blocked morphine analgesia while $G_i\alpha 1$ probes had no significant effect, while M6G analgesia was blocked by ODNs targeting $G_i\alpha 1$ but not $G_i\alpha 2$. These results were exactly paralleled in mice, where it was found that both supraspinal and spinal morphine analgesia was blocked by $G_i\alpha 2$ antisense but unaffected by $G_i\alpha 1$

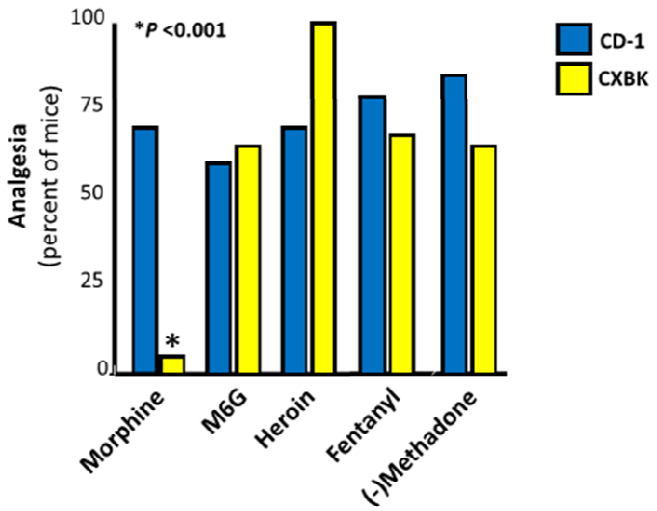
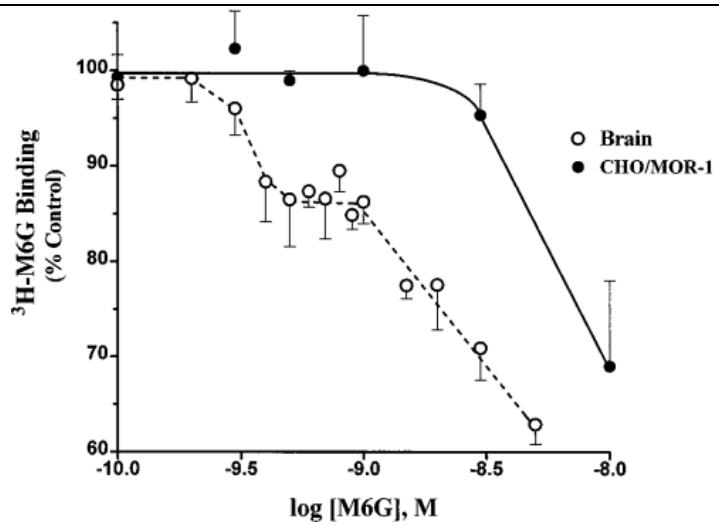


Figure 5: CXBK sensitivity to opioid analgesics.
Approximately equipotent doses of various opioids were administered to wildtype CD-1 or spontaneously mu-deficient CXBK mice. Despite loss of morphine analgesia systemically (shown here) and supraspinally (see **Figure 1**), analgesia produced by a number of other drugs classically considered "mu" analgesics was fully intact in these animals. Reproduced from Pasternak and Pan, 2013; adapted from data from Rossi et al 1996 and Chang et al 1998.

antisense, while the converse was true for M6G (Standifer, Rossi, & Pasternak, 1996). Additionally, both supraspinal and spinal M6G analgesia was reduced by $G_{x/z}\alpha$ antisense, which had no effect on morphine at either site.

Still more evidence for independent receptor mechanisms for morphine and M6G came from experiments with the spontaneously mu-deficient CXBK mice. As previously noted, supraspinally and systemically administered morphine analgesia is profoundly impaired in these animals; however, a survey of other "mu" analgesics displayed strikingly different sensitivity in the CXBK mouse. M6G i.c.v. analgesia was completely intact, as was heroin (s.c.), the heroin metabolite 6-acetylmorphine (i.c.v.), and the extremely potent and clinically used fentanyl (G C Rossi, Brown, Leventhal, Yang, & Pasternak, 1996); Figure 5). Methadone analgesia was also found to be unaffected (Chang, Emmel, Rossi, & Pasternak, 1998).



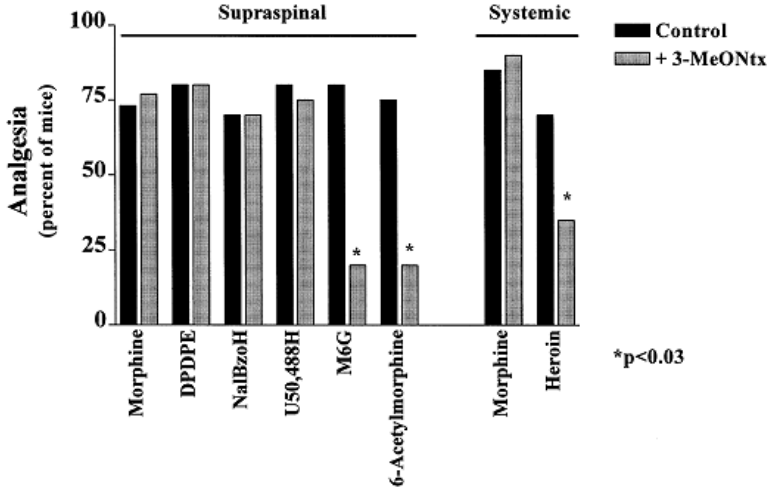


Figure 6: ^{3}H-M6G binding and selective antagonism of M6G and heroin analgesia by 3-methoxynaltrexone.
(Top) M6G competition of ^{3}H-M6G binding in calf brain homogenate reveals the presence of high and low affinity sites; however, in a cell line stably expressing MOR-1, only the lower affinity site is observed. Reproduced from (Brown, Yang, Ouerfelli, et al., 1997). **(Bottom)** At low doses, 3-methoxynaltrexone selectively antagonizes supraspinal M6G and 6-acetylmorphine analgesia and systemic heroin analgesia. Reproduced from (Brown, Yang, King, et al., 1997).

Scatchard analysis of ^{3}H-M6G binding in calf striatum reveals the presence of a very high affinity but very low abundance site (K_D = 68 pM, B_{max} = 6.5 fmol/mg of protein), in addition to a somewhat lower affinity, but higher abundance site (K_D = 1.9

nM, B_{max} = 82 fmol/mg of protein) appearing to correspond to the traditional mu opioid receptor on the basis of competition studies(Brown, Yang, Ouerfelli, et al., 1997). The high affinity component varied in abundance across brain regions, comprising as much as 25% of frontal cortex and striatum binding by homologous competition with unlabeled M6G, while appearing absent entirely from the thalamus, which expresses very high levels of the traditional mu receptor. Furthermore, the site was not present in CHO cells stably expressing the MOR-1 gene. Importantly, 3-methoxynaltrexone, which competed ^{3}H morphine with only modest affinity, also showed biphasic competition of ^{3}H-M6G with a high affinity and low affinity compenent. Further behavioral studies showed that 3-methoxynaltrexone antagonized i.c.v. M6G and 6-acetylmorphine and systemic heroin analgesia an order of magnitude more potently than morphine (Brown, Yang, King, et al., 1997).

Following the antisense evidence supporting the existence of a separate receptor mediating heroin and M6G analgesia which did not depend on exon 1-associated variants, several groups tested M6G in different MOR exon 1 knockout mouse models with conflicting results. Initial attempts by the Uhl group found that both heroin and M6G analgesia were lost in the an MOR-1 exon 1 knockout model and suggested that there was, in fact, no support for an independent receptor mechanism (Kitanaka, Sora, Kinsey, Zeng, & Uhl, 1998). However, a second MOR-1 exon 1 knockout animal was generated in the Pintar lab sparing a large portion of the promotor region spanning upstream of exon 1 which had been knocked out by the Uhl targeting vector (A. G. P. Schuller et al., 1999). In these animals, morphine was completely inactive systemically at doses >19-fold higher than its analgesic ED_{50}, while M6G and heroin retained full analgesic efficacy with only 2-3-fold shift in ED_{50} (Figure 7). Furthermore, M6G analgesia was still sensitive to mu antagonists as well as exon 1 antisense

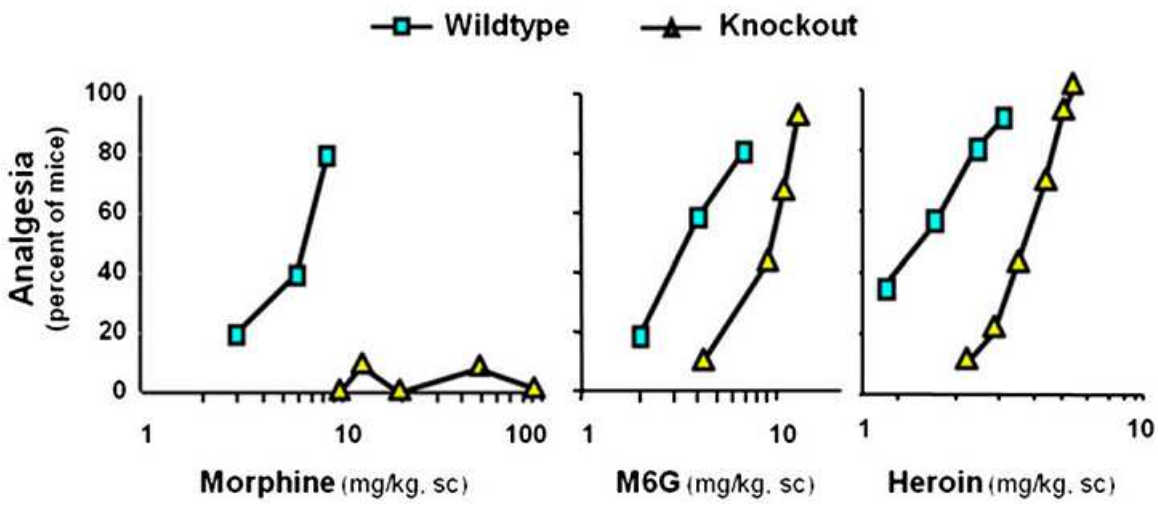


Figure 7: Morphine, M6G and heroin analgesia in Pintar's MOR exon 1 knockout mouse model.
In a tail flick assay, morphine analgesia is completely lost at doses >19-fold higher than its analgesic ED_{50}. Consistent with antisense studies, M6G and heroin analgesia retains full efficacy and dose response curves are only slightly shifted, 2-3-fold. Reproduced from (G. W. Pasternak & Pan, 2013) based on data from (A. G. P. Schuller et al., 1999).

treatment, and ^{3}H-M6G binding was present but at low levels, consistent with retention of the low abundance site wildtype brains. Finally, RT-PCR using probes spanning exons 2 and 3 confirmed the presence of opioid receptor transcripts which lacked exon 1.

Mu Opioid Receptor Splice Variants

C-terminal Splice Variants

Mu opioid receptor gene homologues evolved early in vertebrate evolution, appearing first in teleosts as a gene comprised of 5 exons, each encoding a portion of the 7 transmembrane domains that make up the receptor(Herrero-Turrion & Rodríguez, 2008). Beginning in zebrafish, the introns separating exons 3-5 disappear to form a gene of 3 exons, while a 4th exon is first seen in the chicken. This 4 exon structure is conserved across amphibian, reptile, and mammalian species, and share >71% amino acid identity. The first exon encodes the extracellular N-terminal region as well as

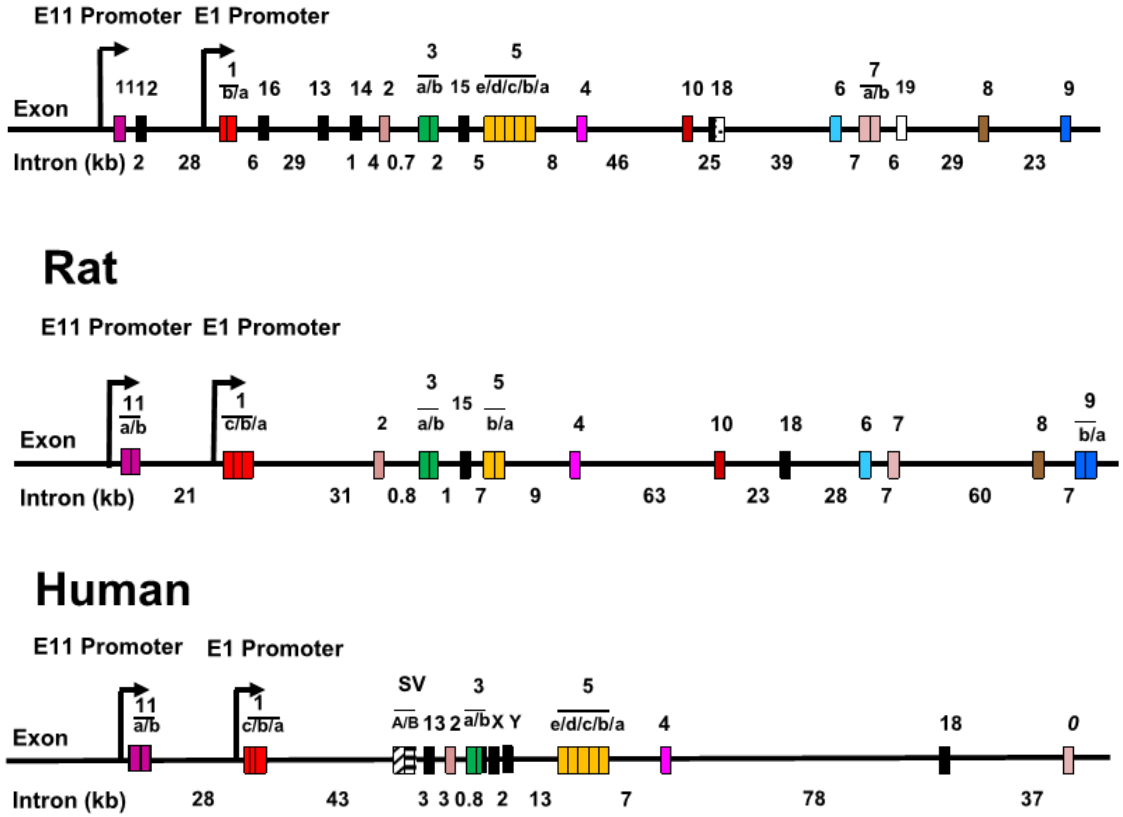


Figure 8: Gene Structure of Mouse, Rat, and Human Mu Opioid Receptor (OPRM-1). OPRM-1 undergoes extensive splicing, and exon structure is largely conserved across mammalian species. Boxes indicate exons and arrows indicate promoters. Genomic distances are indicated but are not to scale. Reproduced from (G. W. Pasternak & Pan, 2013).

the first transmembrane domain, with exons 2 and 3 encoding the remaining 6 transmembrane domains and exon 4 encoding the intracellular C-terminal tail.

However, predictions that the mu opioid receptor underwent alternative mRNA splicing were correct, and in the last 2 decades a dizzying array of additional exons and dozens of mu opioid receptor variants have since been discovered (Figure 8 - 10; L. Pan et al., 2005; Y. X. Pan et al., 1999; Y. Pan et al., 2005; Ying-Xian Pan et al., 2001; D. A. Pasternak et al., 2004; Xu, Xu, Rossi, Pasternak, & Pan, 2011). The first series of variants

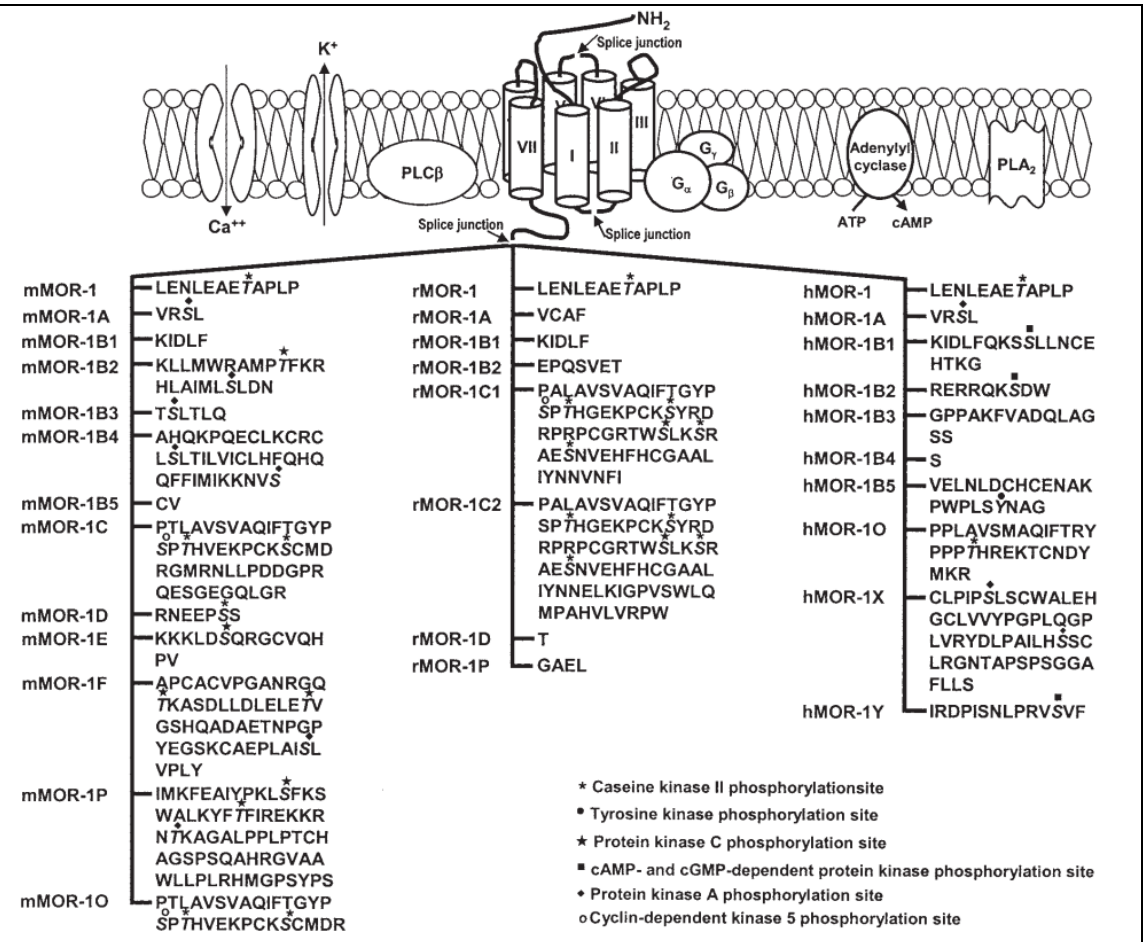
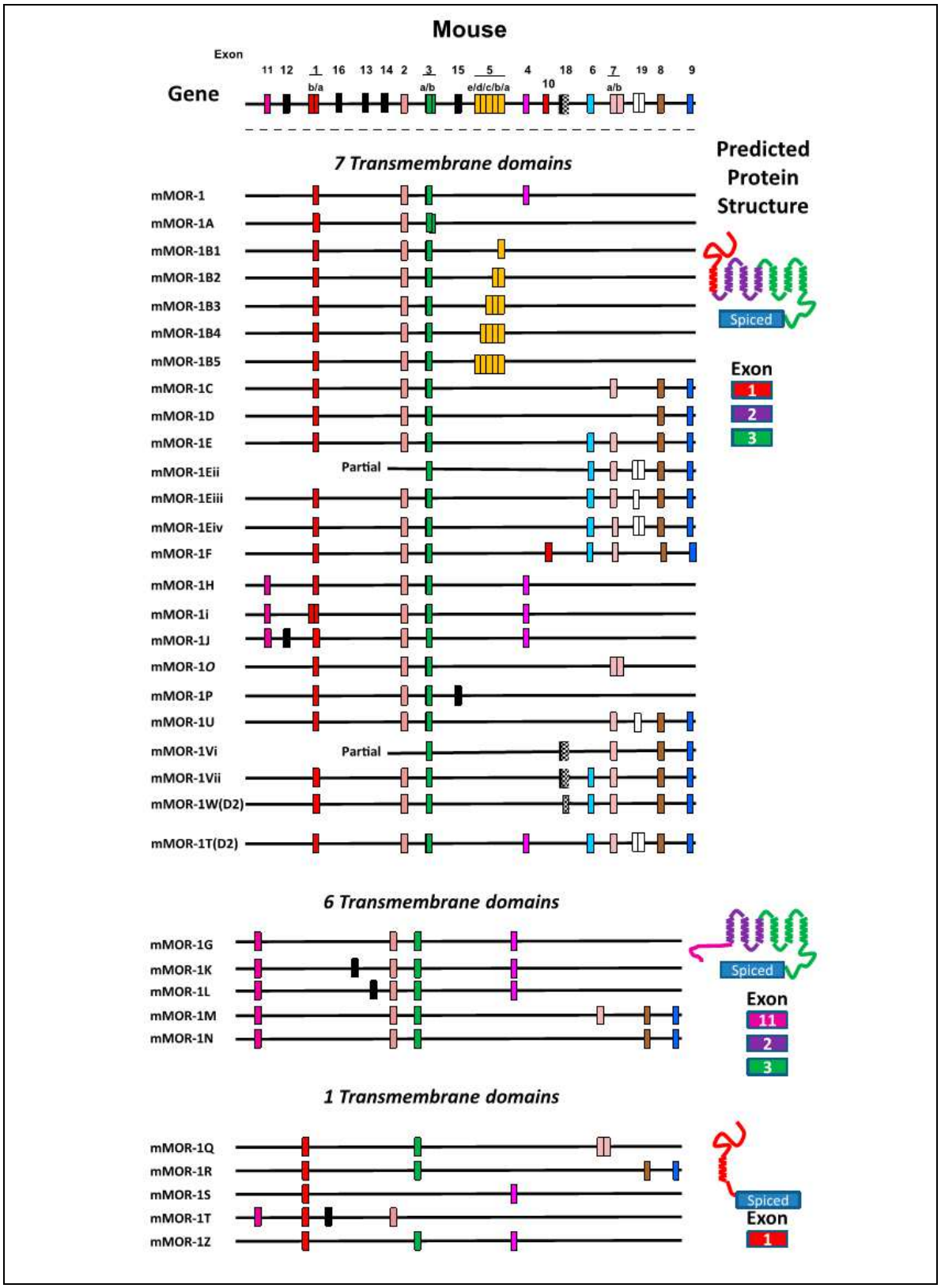


Figure 9: C-terminal Splice Variants of OPRM-1.
Mouse, rat, and human c-terminal splice variant amino acid sequences are shown with symbols indicating predicted phosphorylation sites. Given the importance of phosphorylation in recruitment of b-arrestin, these differences are likely to have significant consequences on G-protein independent signaling and agonist bias. Reproduced from (Ying-Xian Pan, 2005).

discovered differ at their 3' ends, where different exons encode for C-terminal tails. These differ dramatically in both length as well as residues predicted to be subjected to post-translational modification (Figure 9). These differences are likely to have profound implications for receptor pharmacology following agonist binding, as the C-terminal tail is believed to recruit intracellular proteins to the receptor signalosome, altering

Figure 10: **Mouse OPRM-1 splice variants and protein product.**
Splice variants can be sorted into 3 groups – those that produce a full-length, 7TM receptor; variants producing truncated 6TM variants lacking the N-terminus and first TM domain but expressing TMs 2-7 and a C-terminal tail; and variants encoding a single TM domain. Reproduced from (G. W. Pasternak & Pan, 2013).


Mouse
Exon
Gene
11 12 1 16 13 14 2 3 15 5 4 18 6 7 19 8 9
10
b/a
a/b
e/d/c/b/a
a/b
7 Transmembrane domains
Predicted Protein Structure
mMOR-1
mMOR-1A
mMOR-1B1
mMOR-1B2
mMOR-1B3
mMOR-1B4
mMOR-1B5
mMOR-1C
mMOR-1D
mMOR-1E
mMOR-1Eii
mMOR-1Eiii
mMOR-1Eiv
mMOR-1F
mMOR-1H
mMOR-1i
mMOR-1J
mMOR-1O
mMOR-1P
mMOR-1U
mMOR-1Vi
mMOR-1Vii
mMOR-1W(D2)
mMOR-1T(D2)
Partial
Partial
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6 Transmembrane domains
mMOR-1G
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mMOR-1L
mMOR-1M
mMOR-1N
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Exon
11
2
3
1 Transmembrane domains
mMOR-1Q
mMOR-1R
mMOR-1S
mMOR-1T
mMOR-1Z
Spiced
Exon
1

signaling and trafficking (Georganta, Agalou, & Georgoussi, 2010; Venkatakrishnan et al., 2013). Indeed, recycling of the receptor back to the cell surface following agonist-induced internalization is facilitated by the presence of an endocytic recycling sequence that is notably absent from several splice variants such as MOR-1B, D, and E. These variants show significantly reduced recycling of these mutants relative to MOR-1 (Tanowitz, Hislop, & von Zastrow, 2008).

Finally, phosphorylation of the C-terminal tail has been shown to be involved in the recruitment of arrestins, proteins involved not only in the desensitization of receptors but also in G-protein independent signaling (Nobles et al., 2011; Reiter, Ahn, Shukla, & Lefkowitz, 2012; Xiao, Shenoy, Nobles, & Lefkowitz, 2004). b-arrestin 2 knockout mice show greater analgesia following morphine treatment than wildtype animals (Bohn et al., 1999), yet display reduced development of tolerance, constipation, and respiratory depression (Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000; Raehal, Walker, & Bohn, 2005).

As different agonists stabilize slightly different activated conformations of GPCRs with differing affinity for arrestins and G-proteins, these agonists will also signal via G-protein dependent and independent pathways to a greater or lesser degree despite binding the same receptor. This phenomenon, termed “Biased Agonism,” has recently become an area of intense research (Lohse & Hoffmann, 2014), but to date little is known about the effect of alternative splicing, C-terminal tail phosphorylation sites, and cell-type specific effects on receptor trafficking and G-protein independent signaling mechanisms.

N-terminal Splice variants

In addition to the C-terminal splice variants which feature identical binding pockets but differ in their tail region, a second group of MOR splice variants was

identified by Pan et al in 2001 which help explain the remarkable retention of M6G and heroin analgesia in Pintar's MOR exon 1 knockout animals despite total loss of morphine analgesia. Associated with exon 11, an exon found a distant 30kb upstream of exon 1 with its own independent promoter region, a subset of these splice variants skip exon 1 entirely, producing N-terminally truncated product with only 6 transmembrane domains (Figure 10). Despite loss of this first TM domain, these truncated proteins are expressed in a region-specific manor throughout the brain and spinal cord, albeit at lower levels than the full length MOR-1 (Abbadie, Pan, & Pasternak, 2004; Ying-Xian Pan et al., 2001).

Despite perceptions that these truncated receptors were unlikely to be functionally active as they fail to conform to the canonical 7TM architecture shared by the GPCR family, generation of an MOR exon 11 knockout animal confirmed the physiological relevance of the 6TMs (Ying-Xian Pan et al., 2009). These animals retain the majority of their full length MOR receptors, with approximately 20% lower ^{3}H-DAMGO B_{max} observed due to loss of 7TMs which include exon 11 as well as exon 1 (Figure 10). Behaviorally, morphine and methadone retain full efficacy in these animals without significant loss of analgesic potency; in stark contrast, drugs such as heroin, fentanyl, and M6G showed dramatic shifts in analgesic potency (>5, >9, and 21-fold, respectively; Figure 11), consistent with an important role of exon 11-associated splice variants in their actions. Taken together with the results antisense mapping and reciprocal MOR exon 1 knockout animal from Pintar's group which expresses only truncated 6TM but not 7TM MOR splice variants, the evidence overwhelmingly supports a model M6G and heroin act not only at the traditional mu receptors which mediate morphine's effects, but also a pharmacologically and genetically distinct population of receptors involving truncated 6TM receptors.

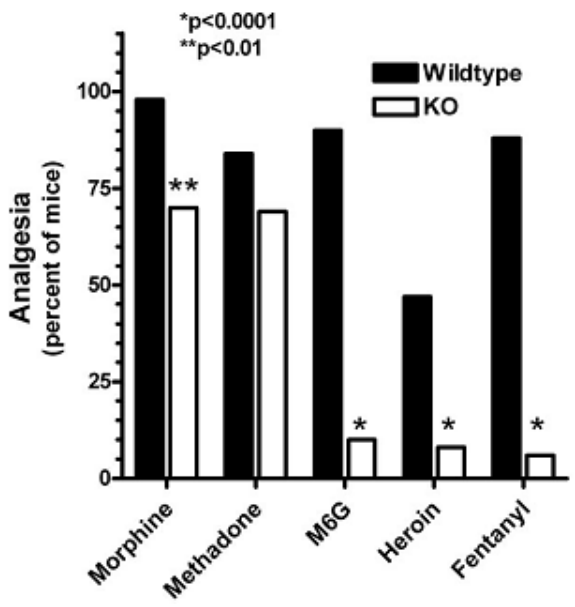


Figure 11: **Selective loss of opioid analgesia in MOR exon 11 knockout animals.** Morphine and methadone analgesia was only slightly shifted in MOR exon 11 knockout relative to wildtype controls. However, M6G, heroin, and fentanyl showed dramatic loss of analgesic potency, consistent with a critical role of exon 11-associated splice variants in mediating their analgesia. Reproduced from (Ying-Xian Pan et al., 2009).

Recent Insights into Opioid Receptor Structure and Function

The crystal structures for all 4 opioid receptors were recently solved by the labs of Brian Kobilka and Ray Stevens through the use of cocrystalized antagonists and T4 lysozyme fusions in the intrinsically disordered 3rd intracellular loop of the receptors, providing an evolutionary leap forward in our understanding of molecular recognition and selectivity features of the receptors (Granier et al., 2012; Manglik et al., 2012; A. Thompson et al., 2012; Wu et al., 2012). Mu receptors were crystallized in a multimeric form, consistent with numerous studies supporting receptor homo- and hetero-dimerization and conformational crosstalk between opioid receptors and other GPCRs (Alfaras-Melainis, Gomes, Rozenfeld, Zachariou, & Devi, 2009; Cussac et al., 2012; Cvejic & Devi, 1997; Gomes et al., 2013; Jordan & Devi, 1999; Kabli, Fan, O'Dowd, & George,

2014; Y-X Pan, Bolan, & Pasternak, 2002; Rozenfeld & Devi, 2007; Rutherford et al., 2008; Sarkar, Sengupta, Zhang, Boyadjieva, & Murugan, 2012); however, it is too early to say whether the interactions observed in the crystal structures represent a physiologically relevant protein-protein interaction interface or are artifacts of the unnatural conditions used to grow crystals for x-ray diffraction studies (Roth BL, personal communication). Future crystal structures examining opioid receptors in complex with agonists, G-proteins, and b-arrestin will further elucidate the mechanics of opioid receptor signaling and advance attempts at rational drug design, especially in designing biased ligands.

Interestingly, the 1st transmembrane domain does not make contacts with the ligands in the crystal structure of the mu opioid receptor, raising the question of whether loss of this TM would substantially perturb ligand binding. This appears to be a common structural feature of GPCRs (Venkatakrishnan et al., 2013). Further studies are needed to determine whether the 6TM receptors are in fact capable of binding drug or activating downstream effectors on their own, or whether they require a partner to function once at the cell surface.

Chapter 2: Materials and Methods

Chemicals: Drugs were obtained from the Research Technology branch of the National Institute on Drug Abuse (Rockville, MD), Tocris Bioscience (Bristol UK), or Caymen Chemical (Ann Arbor, MI). [^{35}S]-GTPγS and Na^{125}I were purchased from Perkin Elmer (Waltham, MA). Miscellaneous chemicals and buffers were obtained from Sigma Aldrich (St. Louis, MO). IBNtxA and ^{125}I-BNtxA were synthesized and structures confirmed as previously described (Majumdar, Burgman, et al., 2011).

In Vivo Assays

Animals: C57Bl/6 mice (24–37 g) were purchased from Jackson Laboratories or Charles River Laboratories. MOR Exon 11 knockout animals were derived as previously described (Pan et al 2009) and backcrossed at least 10 generations on a C57Bl6/J background. Double exon 1/exon 11 knockout mice (E1/E11 KO) on a mixed 129-C57BL/6 background were generated as previously reported (Lu et al., 2015). Exon 1 MOR-1, KOR-1, DOR-1, ORL$_1$, and triple KO (TKO) were generated in the laboratory of John Pintar (Zhang et al 1998, Schuller et al 1999, Zhu et al 1999, Clarke 2002) and were maintained on an inbred 129S6 background. Animals were given at least 1 week washout period after receiving a drug before repeat testing.

Male Sprague Dawley rats were obtained from Charles River Laboratories. All rats were maintained on a 12-h light/dark cycle with Purina rodent chow and water available ad libitum and housed in groups of two until testing. All animal studies were approved by the Institutional Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

All rats and mice were maintained on a 12-h light/dark cycle with Purina rodent chow and water available ad libitum. Mice were housed in groups of five until testing.

All animal studies were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center, Weill Cornell Medical College, or UMDNJ-RWJMS and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility.

Analgesia Assays: Analgesic dose response curves were generated using a radiant heat tail-flick paradigm as previously published with a Ugo Basile 37360 tail flick unit (Varese, Italy). A maximum cutoff latency of 10 seconds was employed to minimize tissue damage and data is presented as % Maximum Possible Effect (%MPE) according to the formula: %MPE = [(Observed latency - Baseline latency) / (10s - Baseline latency)]. Similar results were obtained when data was analyzed quantally with analgesia defined as a doubling of baseline latency. The hot plate assay was performed at 55 °C (Ugo Basile). The time(s) elapsing to the first pain response (hind paw licking or jumping) was scored. A maximal latency of 30 s was used to minimize any tissue damage. ED_{50} values were determined using nonlinear regression analysis where indicated (Graphpad Prism, La Jolla, CA) and curves were compared using extra-sum of squares F-test. Where indicated, group means for individual doses were compared using 2-way ANOVA and post-hoc Bonferroni multiple comparisons test.

For compounds injected subcutaneously (s.c.), analgesia was tested 30 min post-injection at peak effect. Compounds delivered intracerebroventricularly (i.c.v.) were performed as previously described (Haley and McCormick, 1957). Briefly, the mice were anesthetized by isoflurane. A small incision was made, and compounds (2 ul/mouse) were injected using a 10 uL Hamilton syringe fitted to a 27 gauge needle. Injections were made into the right lateral ventricle at the following coordinates: 2 mm caudal to bregma, 2 mm lateral to sagittal suture, and 2 mm in depth. Mice were tested for

analgesia 10 minutes following i.c.v. injections. DPDPE, U50, 488, and clonidine were also administered intrathecally (i.t.) via lumbar puncture (1ul) as previously described (Paul, Standifer, et al JPET 1989; Hylden and Wilcox, 1980). Mice were tested for analgesia 15 minutes after i.t. injections.

Open Field Locomotor Activity: Open field locomotor activity was obtained in a MedAssociates ENV-510 activity chamber (St Albans, VT) using MedAssociates Activity Monitor software. Animals were habituated to the testing room for at least an hour prior to testing each day. Animals were injected s.c. with clonidine and immediately placed in the open field box for 60 min, followed by saline the following day; for buprenorphine, saline was tested on day 1 and buprenorphine on day 2. Total distance traveled as well as distance travelled in 5 min bins were compared using a repeated measures ANOVA followed by Bonferroni's multiple comparisons test.

Conditioned Place Preference: The testing apparatus consisted of two compartments of equal size separated by a wall with a guillotine-style door (ENV-512 insert; MedAssociates). One compartment was surrounded by white walls and had a rod floor, and the other had black walls and a grid floor. Infrared photobeams lining the floor tracked the location of the mouse at all times. Animals were habituated to the environment for 3 h for each of 2 d before testing and for 1 h on each conditioning session. Baseline preferences were determined on the preconditioning test day by letting animals explore both sides freely for 20 min, and the side in which they initially spent more time in was assigned to saline in the place preference study. Animals were injected on alternating days for 8 d with either drug or saline and restricted to one compartment for 20 min. On the postconditioning testing day, animals were placed in the side paired with saline and allowed to freely explore both compartments for 20 min. The time spent in each compartment postconditioning was calculated and subtracted

from the amount of time spent in each compartment preconditioning to determine the change in each animal's preference attributable to conditioning.

Conditioned Place Aversion: Conditioned place aversion was assessed using an unbiased 2-chamber conditioned place preference insert in the activity chamber (MedAssociates ENV-510) and the animals' position monitored using Activity Monitor software. Animals were habituated to the testing room for at least an hour prior to testing each day. On day 1, animals were allowed to freely explore both sides of the apparatus. On days 2-4, animals were injected s.c. with saline and immediately placed into one side off the apparatus for 20 minutes; in the afternoon, they were injected s.c. with 5mg/kg U50,488 which was paired with the opposite side. Animals were randomly assigned to receive drug paired with one side and saline in the other such that 50% of the animals were conditioned to associate the drug with each side. On day 5, animals were again allowed to freely explore both sides of the apparatus. The amount of time spent in each compartment post-conditioning vs. pre-conditioning was compared using a 2-way repeated measures ANOVA followed by a post-hoc Bonferroni's multiple comparisons test (Graphpad Prism). The total distance traveled by each animal after receiving drug on each of the 3 conditioning days was also recorded and normalized to the distance travelled after receiving saline that morning.

Gastrointestinal Motility Assay: Gastrointestinal transit was measured as previously described (Pan et al., 2009). Briefly, animals were injected with either saline or buprenorphine and 10 minutes later received a charcoal meal (10% charcoal and 2.5% gum tragacanth in distilled water) by gavage. 30 minutes after administration of the charcoal meal, animals were sacrificed by cervical dislocation and the distance travelled by the charcoal meal was measured and expressed as a fraction of the total distance from the pyloric sphincter to the cecum.

Respiratory Depression Assays: Respiratory rate was assessed in awake, freely moving, adult male CD1 mice with the MouseOx pulse oximeter system (Starr Life Sciences). Each animal was habituated to the device for 30 min and then tested. A 5-second average breath rate was assessed at 5-min intervals. A baseline for each animal was obtained over a 25-min period before drug injection, and testing began at 15 min post-injection and continued for a period of 35 min. Groups of mice (n = 5) were treated s.c. with either morphine (20 mg/kg) or IBNtxA (2.5 mg/kg) at doses approximately four times their analgesic ED_{50}. Groups were compared with repeated-measures ANOVA followed by Bonferroni multiple-comparison test.

Tolerance and Dependence Studies (Mice) Tolerance was induced by twice-daily injections with either morphine (6 mg/kg s.c.) or IBNtxA (1 mg/kg s.c.) or through the implantation of free-base morphine pellets (75 mg). Dependence was determined on day 3 after pellet implantation with either IBNtxA (1 mg/kg s.c.) or naloxone (1 mg/kg s.c.) to precipitate withdrawal, and animals were evaluated for signs of diarrhea and jumping.

Cross-Tolerance Assays (Rats) To assess for cross tolerance between IBNtxA and morphine, rats (n=4) were made tolerant to morphine by the subcutaneous implantation of three 75mg pellets of morphine freebase under isoflurane anaesthesia (Yoburn, et al., 1985). A control group (n = 10) was implanted with placebo pellets. Three days after pellet implantation, animals were administered escalating doses of IBNtxA and tested in the tail-flick analgesia assay as above. The following day, animals were administered escalating doses of morphine and tested in the tail-flick analgesia assay as above to confirm that the morphine-pelleted animals were indeed morphine tolerant. At the conclusion of the morphine dose-response assay, all animals were given

naloxone 1mg/kg and observed for wet dog shakes for 15 minutes to confirm that the morphine-pelleted animals were indeed physically dependent on morphine.

Lentiviral Injection: Detailed methods have been previously described (Lu et al., 2015). Lentivirus was generated using constructs with and without the mMOR1G sequence in HEK293T cells. Four µl of the lentiviral particles expressing mMOR1G or vector alone were injected supraspinally on days 1, 3, and 5. All drug testing was carried out between 5 and 14 weeks post viral injection, during which protein expression remains stable.

In Vitro Assays

Binding Studies: ^{125}I-IBNtxA binding assays were carried out in rat brain membrane homogenates prepared as previously described (Majumdar, Grinnell, et al., 2011) at a concentration of 0.5 - 1 mg protein/mL. To prevent binding to traditional opioid receptors, binding was carried out in the presence of mu (CTAP), kappa$_1$ (U50,488H), and delta (DPDPE) blockers at a final concentration of 250 nM each.

^{125}I-IBNtxA and ^{125}I-labeled photoaffinity ligand binding assays were carried out in CHO cells stably expressing mMOR-1, mDOR-1, or mKOR-1 receptors, prepared as described previously (Majumdar, Burgman, et al., 2011), with 3-10 µg protein in a volume of 0.5-1 mL of homogenate.

Nonspecific binding was determined in the presence of levallorphan (1 µM) and specific binding is reported. Binding was carried out for 90 min (equilibrium) at 25^{o}C using 0.5 to 1 ml of homogenate. Glass fiber filters were soaked in 0.5% polyethyleneimine for at least 15 minutes prior to filtration to minimize nonspecific binding to the filters.

Stimulation of ^{35}S-GTPγS binding: [^{35}S]-GTPγS Assays were performed based upon published methods (Selley et al., 1998; Bolan et al., 2004; Pan et al., 2005). Membrane

homogenates from C57 mouse brain (25 µg protein) or CHO cells stably transfected with mMOR, mDOR, or mKOR (50 µg protein) were incubated for 1 hr at 30°C with the indicated drug, ^{35}S-GTPγS (0.05 nM) and GDP in a final volume of 1mL assay buffer containing Tris HCl (50 mM; pH 7.4 at 37°C), $MgCl_2$ (3 mM), EGTA (0.2 mM), NaCl (100 mM), and a protease inhibitor cocktail (leupeptin, bestatin, aprotinin, and peptstatin). GDP concentrations were optimized for each receptor assay: DOR-1 and KOR-1, 10µM; MOR-1, 30µM; brain, 60µM). Nonspecific binding was assessed by the addition of 100µM cold GTPγS. Binding was terminated by vacuum filtration through Whatman GF/C glass fiber filters which were rinsed 3x2mL with cold Tris HCl. Filters were cut out and 3mL of scintillation fluor (Liquiscint, National Diagonistics, Atlanta, GA) was added to each tube and incubated at room temperature for at least 2 hours before being counted on a Packard Tri-Carb TR-2900 liquid scintillation counter.

β-Arrestin-2 Recruitment Assay: β-arrestin-2 recruitment was determined using the PathHunter enzyme complementation assay (DiscoveRx. Fremont, CA) using modified MOR-1 in CHO cells (a gift from DiscoveRx). Cells were plated at a density of 2500 cells/well in a 384-well plate as described in the manufacturer's protocol. The following day, cells were treated with the indicated compound for 90 minutes at 37°C followed by incubation with PathHunter detection reagents for 60 minutes. Chemiluminescence was measured with an Infinite M1000 Pro plate reader (Tecan, Männedorf, Switzerland).

Photoaffinity Labeling

The indicated ^{125}I-labeled photoaffinity ligand was incubated with 0.5mg-2mg protein at a final concentration of 0.3-0.5 nM in a volume of 2mL 50 mM KPO4 (Mu receptors) or 50 mM KPO4/5mM MgSO4 in microcentrifuge tubes for 90 minutes at 25C to reach equilibrium. Tubes were then centrifuged for 10 min at room temperature at 20,000g to separate free ligand and rapidly resuspended in 2mL ice-cold KPO and

transferred to an acrylic cuvette (Nümbrecht, Germany). Photolysis was initiated in a Rayonet photoreactor (RPR-200, Southern New England Ultraviolet Company, Branford CT) equipped with 13 RPR-3500 bulbs for irradiation at 350nm for 5 minutes. The photolysate was then transferred back to a microcentrifuge tube, centrifuged at 4C for 10 min, and the supernatant discarded. The pellet was then solubilized at 4C with either denaturing (RIPA buffer) or nondenaturing (0.2-0.25wt% MNG-3 in 1X Tris buffered saline; Chae et al., 2010) buffer with EDTA-free HALT protease inhibitor cocktail (containing AEBSF HCl, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A; ThermoFisher Scientific, Waltham, MA). The solubilizate was then clarified by centrifugation for 10 min at 4C at 20,000g.

The clarified solubilizate was either directly run on a 10% SDS-PAGE gel (BioRad, Berkeley CA) at 300V for 19 minutes following incubation at room temperature for 30 min with Laemmli sample buffer and 5% b-mercaptoethanol or subjected to further purification. BioRad Precision Plus molecular weight markers were used, with MagicMark XP western blot molecular weight markers. Gels were transferred to PVDF in a BioRad Transblot semi-dry transfer device for 7 min at 2.5A and apposed to film with a Kodak Biomax Intensifying Screen at -80C for exposure from 3 hours – 8 days.

Wheat germ agglutinin (WGA) affinity purification was performed according to the manufacturer's protocol (Vector Labs, Burlingame, CA). Briefly, 25uL of a slurry of WGA-agarose beads was transferred to a microcentrifuge tube containing 20 volumes of nondenaturing buffer and washed 3 times with 20 volumes of fresh buffer solution. The clarified solubilizate was added and rotated for 30 min at room temperature before 3 washes with 30 volumes of fresh nondenaturing buffer. The target was then eluted twice using N-acetylglucosamine elution solution (Vector Labs), run through a 7kDa MWCO Zeba spin desalting column equilibrated with nondenaturing buffer, and

concentrated on a 10kDa NMWL Amicon Ultra 0.5 spin concentrator cartridge to the desired final volume. The final product was incubated at room temperature for 30 min with Laemmli sample buffer and 5% b-mercaptoethanol and separated by SDS-PAGE as above.

Immunoaffinity purification was also performed under both denaturing and nondenaturing conditions, according to manufacturer's protocols. Solubilizate was incubated with rabbit polyclonal antibody directed against the C-terminal tail of the mu opioid receptor (C-20, Santa Cruz Biotechnology, Dallas, TX) according to the manufacturer's suggested dilution relative to the protein added, rotating at 4C for 1hr – overnight. Antibody-Receptor complexes were then pulled down with Protein A-agarose (EzView Red, Sigma Aldrich) for 1-2 hours and equilibrated according to manufacturer's protocols. Beads were washed 2-3 times with 30 volumes buffer until no radioactivity could be detected in the wash with a survey meter. For denaturing conditions, beads were eluted for 30 min with 6.5M urea, 150mM DTT, and 1X Laemmli sample buffer at room temperature and separated by SDS-PAGE as above. For native conditions, beads were eluted 2 times with 100 ug/mL MOR C-20 blocking peptide (Santa Cruz Biotechnology) for 30 min at room temperature. The eluate was then concentrated in a 100kDa NMWL Millipore Biomax spin concentrator.

Blue Native PAGE. Blue native gel electrophoresis was performed according to manufacturer's protocols (Life Technologies, Carlsbad, CA), based on the method of (Schägger & von Jagow, 1991). Briefly, samples were prepared by adding glycerol (10% final concentration) and Coomassie Brilliant Blue G-250 (0.05-0.0625% final concentration). Samples were then loaded onto a 4-16% native gel (Life Technologies) and separated for 105-115 minutes at 150V. Gels for imaging were destained in 40% MeOH/10% Acetic Acid solution for 15 min, followed by 8% Acetic acid for 1hour, then

imaged on a Chemidoc MP (BioRad). Gels for autoradiography were transferred to PVDF and apposed to film as above.

2D Blue Native/SDS-PAGE was performed by excising a lane from a BN-PAGE gel and incubating at room temperature in 1% SDS / 1% β-mercaptoethanol with rocking for 30 min. The highest molecular weight portion of the lane was trimmed to make a 6cm strip for insertion into a Life Technologies NuPage Novex 12% Bis-Tris gel with a 2D well. Gels were run with MOPS-SDS buffer for 1 hour at 200V per manufacturer's protocol, then transferred to PVDF and apposed to film with an intensifying screen as above.

Chapter 3: Characterization of a novel opioid pain reliever, IBNtxA, with a superior side effect profile

Introduction

Tritium is commonly used as a radioisotope for radiolabeling of drugs in binding assays because it is chemically identical to hydrogen and can thus be incorporated into any known drug without altering the binding properties of the parent compound. Since binding assays depend on counting the number of bound radioligand molecules, as specific activity increases, less tissue is required to produce the same number of counts observed for a given receptor site; although carbon 14 can be incorporated into molecules without altering their binding properties as well, the very low specific activity of this isotope prohibits its use for researching sites of low abundance - such as those for neurotransmitters in the brain.

Indeed, poor specific activity of the tritiated and ^{14}C-labeled levorphanol used by Avram Goldstein had thwarted his attempts at identifying the opioid receptors (Goldstein et al., 1971). Likewise, the challenges of discovering opioid receptor subtypes of necessarily lower abundance require higher specific activity radioligands. Of course, as specific activity is proportional to halflife, short lived isotopes must be prepared more frequently. For these reason, the higher specific activity iodine-125 falls into a sweet spot - specific activity of a radioiodinated drug is at least 40-fold greater than a typical tritiated compound, yet once prepared it can be used without substantial decay for over a month. Additionally, after electron capture and transmutation to an excited state tellurium-125, the nuclide releases a gamma ray, permitting the use of a gamma counter rather the environmentally hazardous scintillation fluor required for high efficiency counting of beta particles.

Table 1: Commonly used isotopes in biological research			
Isotope	Halflife	Specific Activity	Decay Emission
3H	12.43 years	28.7 Ci/mmol	Beta particle
14C	5730 year	0.0625 Ci/mmol	Beta particle
125I	59.6 days	2190 Ci/mmol	Gamma ray, X-ray, electrons
35S	87.4 days	1493 CI/mmol	Beta particle
32P	14.3 days	9128 Ci/mmol	Beta particle

For these reasons, Dr. Majumdar set out to make a ^{125}I-labeled radioligands based on the mu opioid agonist oxymorphone, as well as the nonselective opioid receptor antagonists naloxone and naltrexone (Figure 12) – named IBOxyA, IBNalA, and IBNtxA, respectively (Majumdar, Burgman, et al., 2011).

In the early 1970s, the Snyder lab chose the opioid antagonist levallorphan to define nonspecific binding for two important reasons: first, although he was not successful at discovering an opiate receptor, Goldstein reasoned that specific binding must be stereoselective since only one enantiomer was active behaviorally – both enantiomers of levallorphan were available, so it was possible to establish this condition. Second, unlike either Terenius or Simon, the Snyder lab initially used radioligand ^{3}H-naloxone, an antagonist, which therefore bound both active and inactive conformations of the receptor with high affinity; the ideal drug for defining nonspecific binding should also have high affinity for both active and inactive conformations, precluding the use of an agonists such as methadone or levorphanol which possess high affinity only for the active conformation which mediates their effects, but much lower affinity for the inactive conformation (G. W. Pasternak & Snyder, 1975).

The choice of levallorphan decades earlier was fortuitous. Initial binding studies performed on ^{125}IBNtxA used levallorphan to define nonspecific binding, and curiously the parent opioid receptor antagonists naloxone and naltrexone failed to fully inhibit

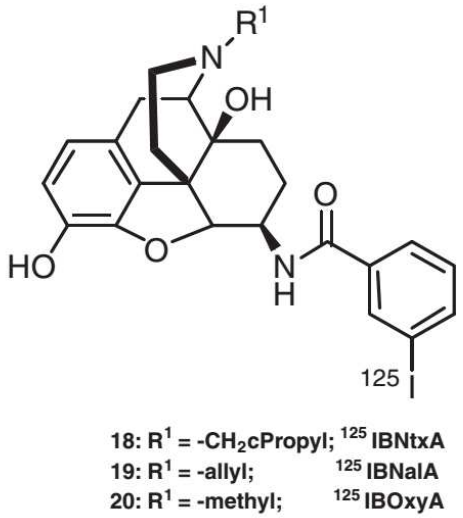


Figure 12: Iodobenzoyl derivatives of Naltrexone, Naloxone, and Oxymorphone. Reproduced from (Majumdar, Burgman, et al., 2011).

binding in mouse brain. This finding hinted at the existence of another, as yet uncharacterized opioid receptor site insensitive to naloxone and naltrexone but sensitive to levallorphan. Using a combination of selective mu, delta, and kappa blockers confirmed that this binding did not correspond to any of the canonical opioid receptors, and binding remained in a triple knockout animal lacking all full length mu, delta, and kappa opioid receptors, confirming that it could not correspond to any of these sites – yet, the high affinity for levallorphan suggested that it was indeed opiate in nature.

Years earlier, a compound called ^{3}H-naloxone benzoylhydrazone (NalBzOH) had been synthesized to study pseudoirreversible mu opioid binding, but in fact demonstrated an additional reversible binding component which was not competed by mu- or delta-selective drugs, and thus deemed kappa-like (Price, Gistrak, Itzhak, Hahn, & Pasternak, 1989). However, the selective kappa ligand U50,488 failed to compete this binding with high affinity, leading to the suggestion that the drug was binding a previously unknown kappa subtype, named kappa$_3$. Indeed, NalBzOH competed the remaining IBNtxA binding with subnanomolar affinity, suggesting a common target.

Surprisingly, binding was found to be lost in a newly generated MOR exon 11 knockout mouse selectively lacking truncated splice variants of the mu opioid receptor, but retaining full length mu receptors, confirming that the binding was definitely related to the mu opioid receptor but did not correspond to the canonical mu sites. After obtaining such compelling binding data *ex vivo*, we sought to characterize the drug *in vivo* to determine the behavioral pharmacology of the drug and its novel target.

Results

IBOxyA and IBNtxA were tested in a radiant heat tail-flick paradigm in mice, under the initial assumption that the compound possessing an N-methyl group based on the agonist, oxymorphone, would be an analgesic, while IBNtxA, with its N-cyclopropylmethyl group known to convey antagonist activity at traditional mu sites, was expected to behave as an antagonist. To our surprise, OxyIBA failed to increase baseline tail flick latencies at any dose tested (not shown), while IBNtxA was a potent analgesic with an ED_{50} of about 0.3 mg/kg (Figure 13A), around 10-fold more potent than morphine in this assay. It was also active in a hot plate model of thermal pain with similar potency (Figure 13B). Also, the drug was active when given orally, although it was around 10-fold less potent via this route (Figure 13C).

Importantly, when we tested IBNtxA in triple knockout animals, there was no significant shift in analgesia, confirming that the analgesia produced was mediated not by the drug's actions at any of the canonical mu, delta, and kappa receptor (Figure 14A). This corresponded perfectly with the binding data showing that mu-, delta-, and kappa-blocker resistant binding was retained in these animals, and suggested that this binding site was indeed responsible for the observed analgesia. Indeed, levallorphan was shown to readily reverse IBNtxA analgesia (Figure 14B; ID_{50} = 0.54 mg/kg s.c.), consistent with

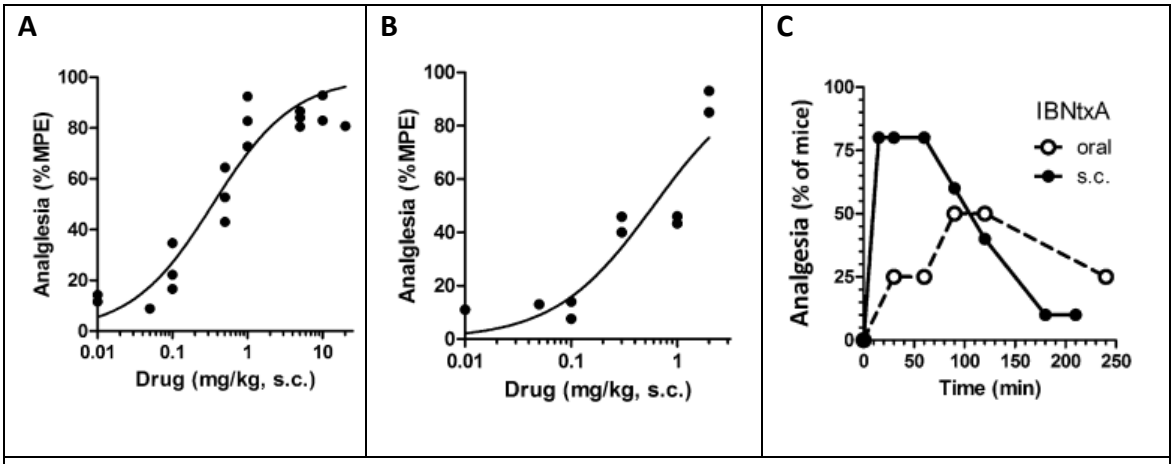


Figure 13: IBNtxA analgesia in wildtype CD-1 mice.
(A) Tail flick analgesia. CD-1 mice were injected s.c. with escalating doses of IBNtxA and were tested 30 minutes later at peak effect in a radiant heat tail flick assay. (B) Hotplate analgesia. Two groups of CD-1 mice (n = 8) were injected s.c. with escalating doses of IBNtxA and were tested independently in a 55 °C hot plate assay. ED_{50} in this assay was = 0.6 mg/kg. (C) Analgesia time course. Groups of mice received IBNtxA administered s.c. (0.75 mg/kg, n = 20) or orally (5 mg/kg by gavage, n = 8) and were tested in a tail flick assay at the indicated times.

its high affinity competition of the non-traditional site in binding assays, while analgesia was much less sensitive reversal by naloxone (ID_{50} = 10.5 mg/kg) than morphine (ID_{50} = 0.01 mg/kg). Finally, as predicted by the binding data, the drug's analgesia was also substantially lost in the MOR exon 11 knockout generated by Dr. Pan (Figure 14C), confirming the critical role of truncated exon 11-associated splice variants in mediating the drug's actions. The reciprocal knockout findings demonstrate that the site mediating IBNtxA analgesia requires truncated exon 11-associated variants but not full length exon 1-associated variants which are lost in the triple knockout animal.

We next sought to investigate the side effect profile of the drug. Opiate overdose can be lethal, especially in illicit settings as recreational users are not monitored for hypoxia or apnea as in a healthcare context. Death occurs primarily due to depression of respiration by MORs expressed in the brainstem, where mu agonists disrupt both the respiratory rhythm as well as the drive to breathe (Pattinson, 2008).

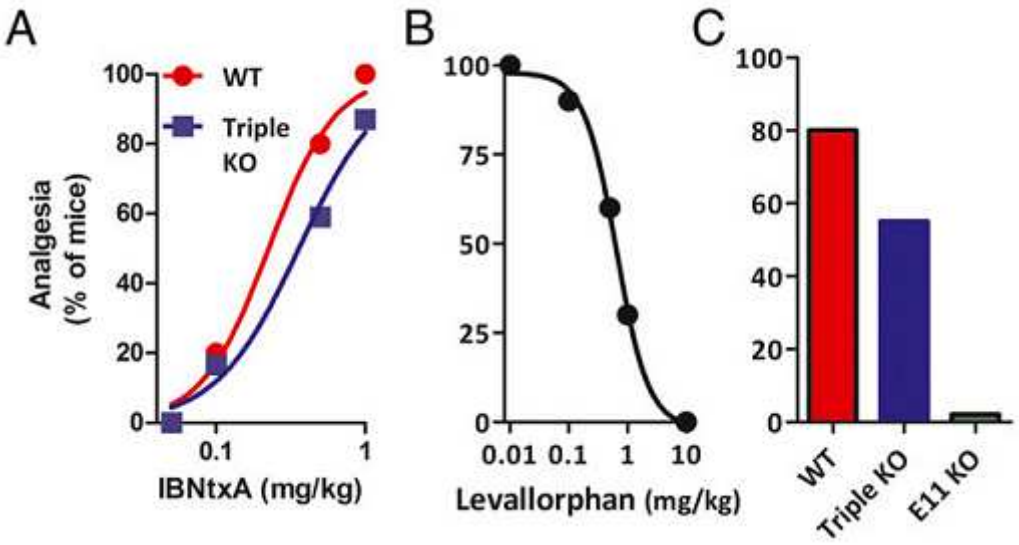


Figure 14: IBntxA analgesia in MOR knockout models and reversal by levallorphan
(A) IBNtxA analgesia in wildtype and triple opioid receptor knockout animals. Groups of mice (n = 10 each) were injected s.c. with escalating doses of IBNtxA and were tested 30 minutes later at peak effect in a radiant heat tail flick assay. Animals were considered "analgesic" when the latency to tailflick was at least 2x their baseline. ED50 values [95% Confidence interavals] were 0.22 mg/kg [0.13 – 0.32] for the wildtype C57 group and 0.39 mg/kg [0.15 – 0.58] for triple knockout mice. (B) Levallorphan antagonism of IBNtxA analgesia. Groups of mice (n ≥ 10) were injected s.c. with IBNtxA (0.75 mg/kg) along with the indicated dose of levallorphan and were tested in a tail flick assay 30 minutes later. Levallorphan potently blocked IBNtxA analgesia with an ID50 value ± SEM of 0.54 ± 0.05 mg/kg. (C) IBNtxA analgesia in MOR Exon 11 knockout animals. IBNtxA analgesia (0.5 mg/kg) was determined in the tail flick assay as above in groups (n = 10 each) of wildtype C57, Triple Knockout, and MOR Exon 11 knockout animals. Although there was no difference between wildtype C57 and triple knockout response, Exon 11 animals expressed significantly lower analgesia relative to both wildtype and Triple KO groups (Fisher exact test).

Animals were injected subcutaneously with saline vehicle, morphine, or IBNtxA at equipotent doses approximately 4-5 times their analgesic ED_{50} and their respiration monitored as the animals moved freely about their cages. As expected, morphine produced a clinically meaningful and profound depression of respiratory rate of approximately 50% lower than their baseline values; IBNtxA did not differ from saline at any time point measured.

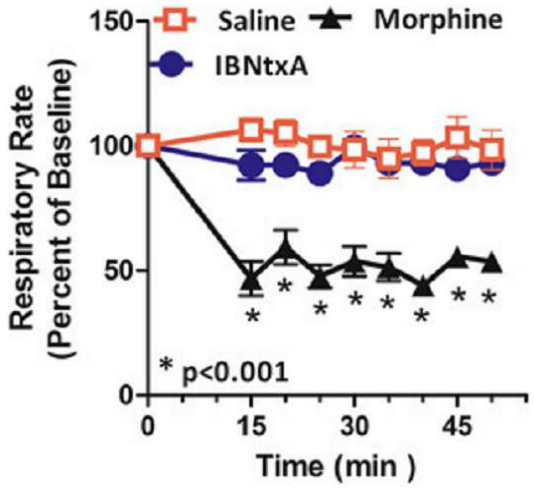


Figure 15: Effects of IBNtxA on respiration.
Animals were randomly assigned to receive saline (n = 5), IBNtxA (2.5 mg/kg, n = 5), or morphine (20 mg/kg, n =5), injected s.c. Each animal's baseline average breath rate was measured every 5 min for 25 min before drug injection, and breath rates after drug injection are expressed as a percent of baseline. IBNtxA did not depress respiratory rate and was not significantly different from saline at any time point, whereas morphine decreased respiratory depression in comparison with both saline and IBNtxA ($P < 0.001$) as determined by repeated-measures ANOVA followed by Bonferroni multiple-comparison test.

One often dose-limiting side effect produced by clinically used opiates is constipation. This effect is mediated primarily by mu and/or kappa receptors expressed in the gut (Culpepper-Morgan, Holt, LaRoche, & Kreek, 1995). Therefore, we investigated whether IBNtxA reduced the gastrointestinal motility of a charcoal meal in mice (Figure 16). Again, we observed the expected dramatic decrease in GI transit produced by morphine at its analgesic ED_{50}. Although some decrease in distance travelled was observed for IBNtxA, the magnitude was far less than that seen with morphine, and exhibited a ceiling effect across doses several times its analgesic ED_{50} suggesting a much more limited ability to depress GI transit (Figure 16A). More importantly, although analgesia was lost in MOR exon 11 knockout mice, the animals still exhibited decreased gastrointestinal motility, suggesting that the effect is off-target and can be dissociated

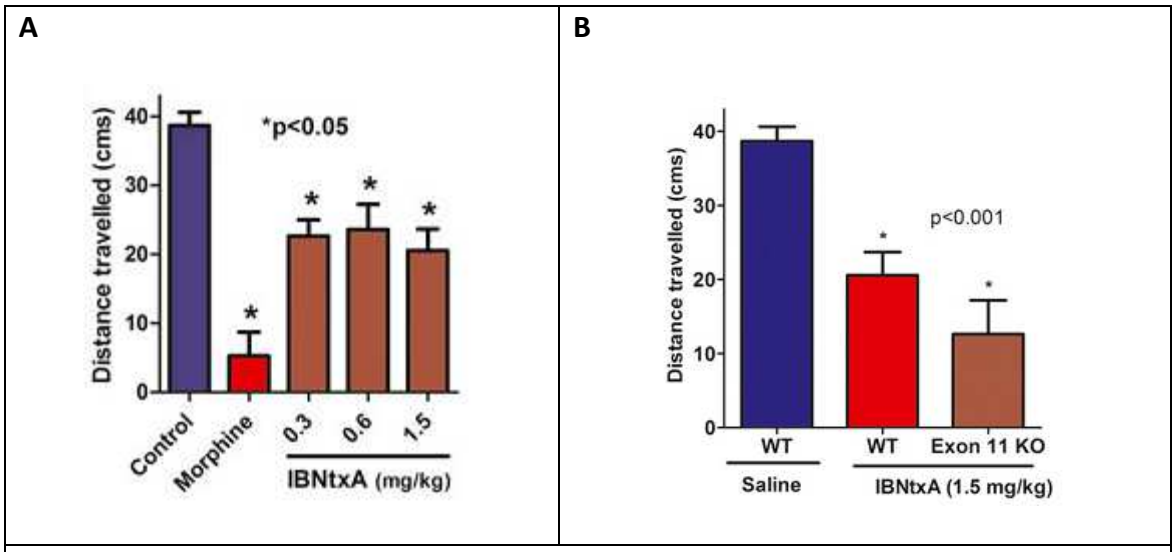


Figure 16: Effect of IBNtxA on gastrointestinal transit.
(A) Groups of mice (n = 10 each) were randomly assigned to receive saline, morphine (5 mg/kg), or IBNtxA (0.3, 0.6, and 1.5 mg/kg) injected s.c. before receiving an oral dose of 0.2 mL of charcoal meal (2.5% gum tragacanth and 10% charcoal in water) by gavage. Animals were sacrificed 30 min later, and the distance traveled by charcoal was measured. IBNtxA lowered transit significantly compared with saline ($P < 0.05$) but less than morphine ($P < 0.05$) as determined by ANOVA followed by Tukey's multiple-comparison test. There was no significant difference between doses of IBNtxA, suggesting a ceiling effect on GI transit to doses up to 3 times the analgesic ED_{50}. (B) Groups of wildtype C57 or MOR Exon 11 knockout mice (n = 10 for each group) were injected with either saline or IBNtxA (1.5 mg/kg) and tested in the GI transit assay as above. IBNtxA reduced GI transit relative to saline in both genotypes ($p < 0.001$,Tukey's multiple comparison test after 1-way ANOVA).

from the drug's analgesia (Figure 16B).

Next, we examined tolerance and dependence to IBNtxA's analgesic effects (Figure 17). As with morphine, chronic dosing of animals with IBNtxA resulted in decreasing effectiveness over time; however, this developed much more slowly. Mice made tolerant to morphine exhibited profound withdrawal signs such as jumping behavior when challenged with a low dose of naloxone, yet animals made tolerant to IBNtxA failed to show jumping behavior significantly different from saline controls when given either low or high doses of either naloxone or levallorphan. Furthermore, animals made highly tolerant to morphine via the implantion of high-dose, slow-release pellets

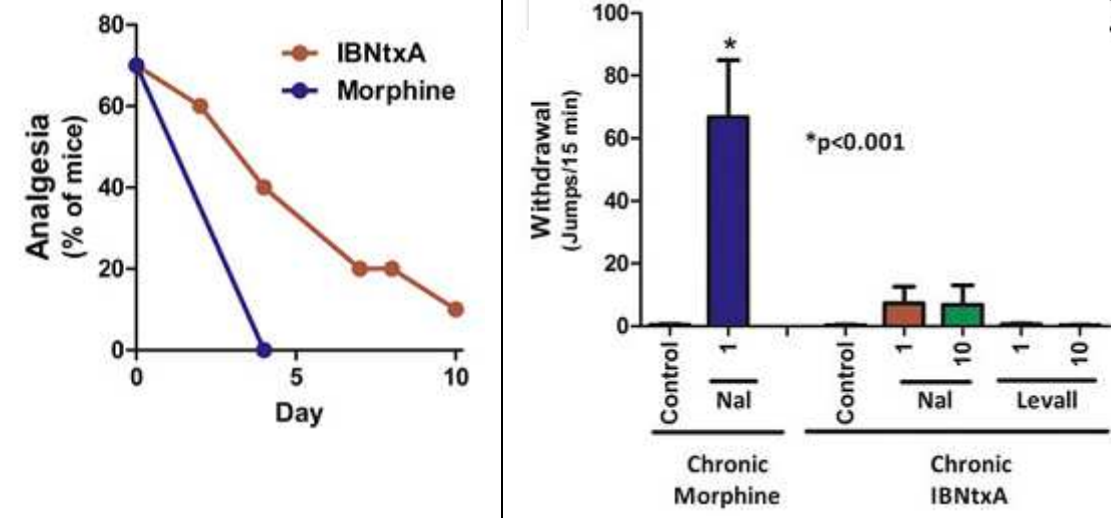


Figure 17: Tolerance and physical dependence to IBNtxA.
(A) Tolerance. Groups of animals (n ≥ 20) were injected s.c. two times daily with equipotent doses of morphine (6 mg/kg) or IBNtxA (1 mg/kg). Analgesia was tested on the indicated days to assess the level of tolerance to each drugs' effects. (B) Physical dependence. Groups of animals (n ≥ 10) were injected twice daily for 10 days with either morphine (10 mg/kg) or IBNtxA (1 mg/kg). Animals were then injected with a challenge dose of saline, naloxone (1 mg/kg, s.c.) or levallorphan (1 mg/kg s.c.) and monitored for jumping behavior, a robust sign of withdrawal, for 15 minutes. Saline produced no jumping in any group, while animals chronically treated with morphine exhibited a profound withdrawal syndrome ($p < 0.001$, Tukey's post-hoc multiple comparisons test after one way ANOVA). Neither naloxone nor levallorphan produced significant withdrawal signs in animals chronically treated with IBNtxA.

showed no cross-tolerance to IBNtxA, lending further support to the case that IBNtxA's target is independent of the traditional mu receptor.

Perhaps the most well-known side effect of mu agonists is euphoria, leading to their recreational use, abuse, and addiction. In a conditioned place preference assay, we sought to determine whether IBNtxA produced rewarding behavior (Figure 18). When animals were trained to associate morphine with one chamber of the apparatus, they developed a strong preference for this side, consistent with its well-established

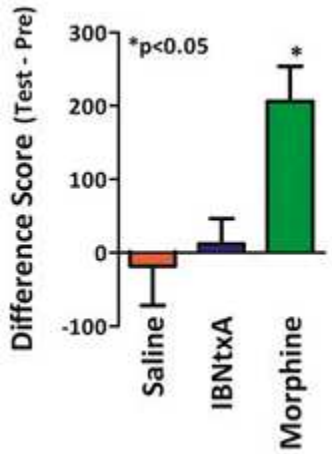
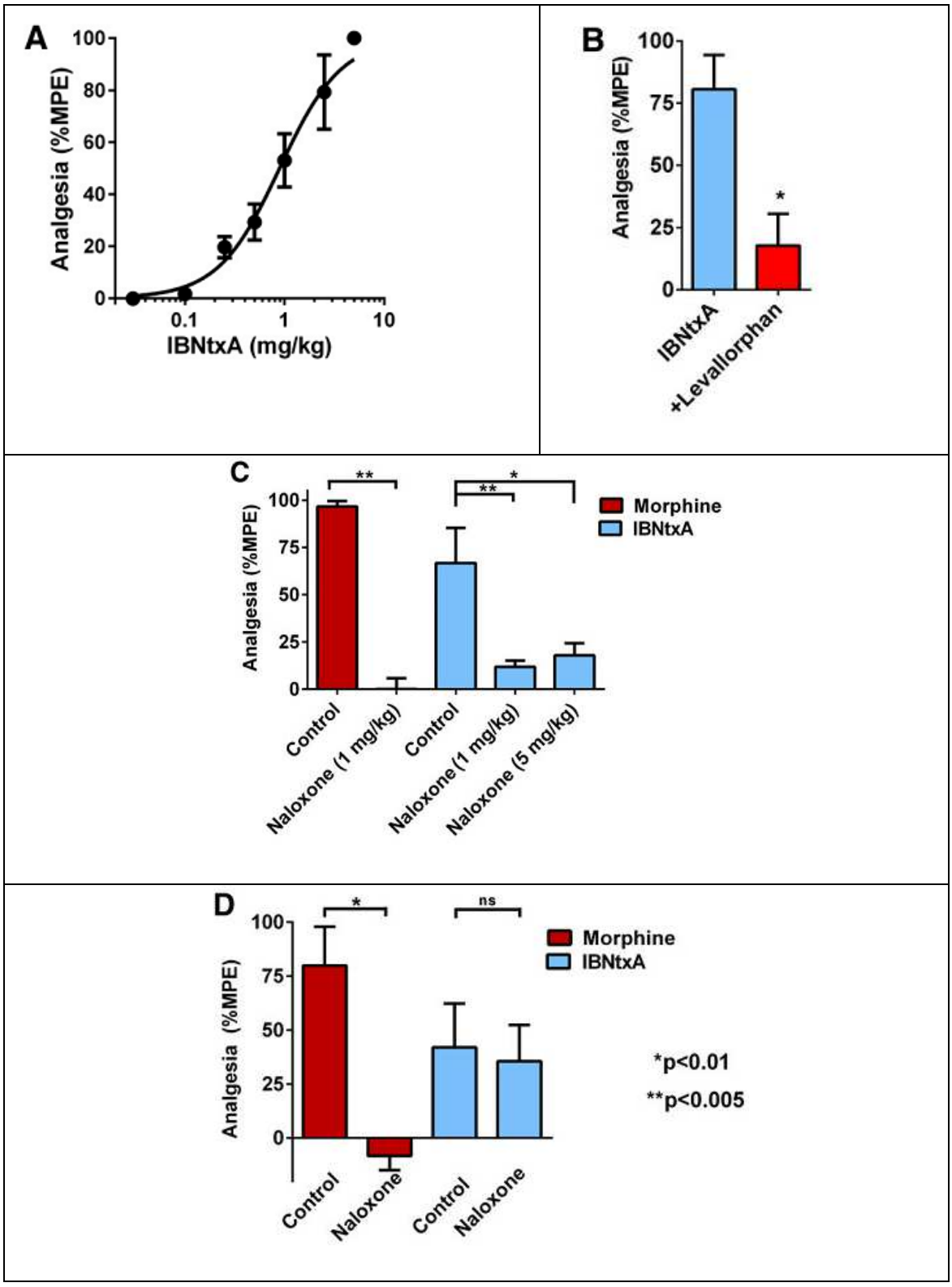


Figure 18: Conditioned place preference experiments with IBNtxA.
Mice were randomly assigned to receive saline (n = 10), IBNtxA (1 mg/kg, n = 10), or morphine (10 mg/kg, n = 13) in a two-compartment conditioned place preference assay. IBNtxA did not produce place preference or aversion on the final test day relative to pre-conditioning chamber preference, and did not differ significantly from saline ($P > 0.05$), whereas morphine showed preference in comparison with both saline and IBNtxA ($P < 0.05$) as determined by Tukey's multiple-comparison test following a one-way ANOVA.

rewarding effects; however, animals who were trained with an equipotent dose (2 times the analgesic ED_{50}) of IBNtxA failed to exhibit either preference for, or aversion to, the drug-paired side relative to saline-treated control animals, suggesting the drug's abuse liability is likely to be substantially improved over morphine.

Although rats and mice both belong to the family *Rodentia* and are both commonly used animal models in laboratory research, their behavioral responses to opiates can differ markedly, meaning that behavioral observations obtained in one species may not necessarily be predictive of human response. For instance, although administration of morphine to humans results in sedation, mice exhibit increased locomotor activity, unlike rats, whose locomotor behavior is depressed. Additionally, although mice, rats, and humans all possess homologous truncated exon 11-associated

Figure 19: IBNtxA analgesia and sensitivity to naloxone in the rat.
(A) Groups of rats(n = 4) were assessed for IBNtxA analgesia at peak effect in three independent experiments (n = 12 total) in a radiant heat tail flick assay. Animals received escalating doses of IBNtxA to generate the analgesic dose response curve. The ED50 was 0.89 mg/kg (95% confidence interval, 0.69–1.2). (B) Groups of rats (n = 8) received IBNtxA (2 mg/kg) and were tested for analgesia after 30 minutes, immediately given levallorphan (1 mg/kg s.c.),and tested 20 minutes later. (C) Groups of rats (n = 4) received either morphine (8 mg/kg s.c.) or IBNtxA (2 mg/kg s.c.) immediately after the injection of saline or the indicated dose of naloxone. Naloxone significantly blocked morphine analgesia (Bonferroni multiple comparison test, $P < 0.0001$) as well as IBNtxA ($P < 0.005$ at 1 mg/kg, $P < 0.01$ at 5 mg/kg). (D) To assess the ability of a low dose of naloxone to reverse analgesia groups of rats were injected with equivalent analgesic doses of morphine 8 mg/kg s.c. (n = 4) or IBNtxA 2 mg/kg s.c. (n = 5). Animals were tested in the tail-flick assay after 20 minutes for morphine and 30 minutes for IBNtxA. They were then immediately administered naloxone (0.1 mg/kg s.c.) and tested again in the tail-flick assay 20 minutes later. Results are reported as percentage of maximum possible effect (%MPE). The results for morphine revealed a significant reduction by naloxone (paired t test, P , 0.005). There was no significant (ns) naloxone effect against IBNtxA.


A
Analgesia (%MPE)
100
80
60
40
20
0
0.1
1
10
IBNtxA (mg/kg)
B
Analgesia (%MPE)
100
75
50
25
0
*
IBNtxA
+Levallorphan
C
Analgesia (%MPE)
100
75
50
25
0
**
**
*
Morphine
IBNtxA
Control
Naloxone (1 mg/kg)
Control
Naloxone (1 mg/kg)
Naloxone (5 mg/kg)
D
Analgesia (%MPE)
100
75
50
25
0
*
ns
Morphine
IBNtxA
Control
Naloxone
Control
Naloxone
*p<0.01
**p<0.005

variants of the OPRM-1 gene which encodes the mu opioid receptor, the splicing patterns of the rat more closely mimic that of humans (Xu et al., 2011). We therefore examined the pharmacology of IBNtxA in the rat.

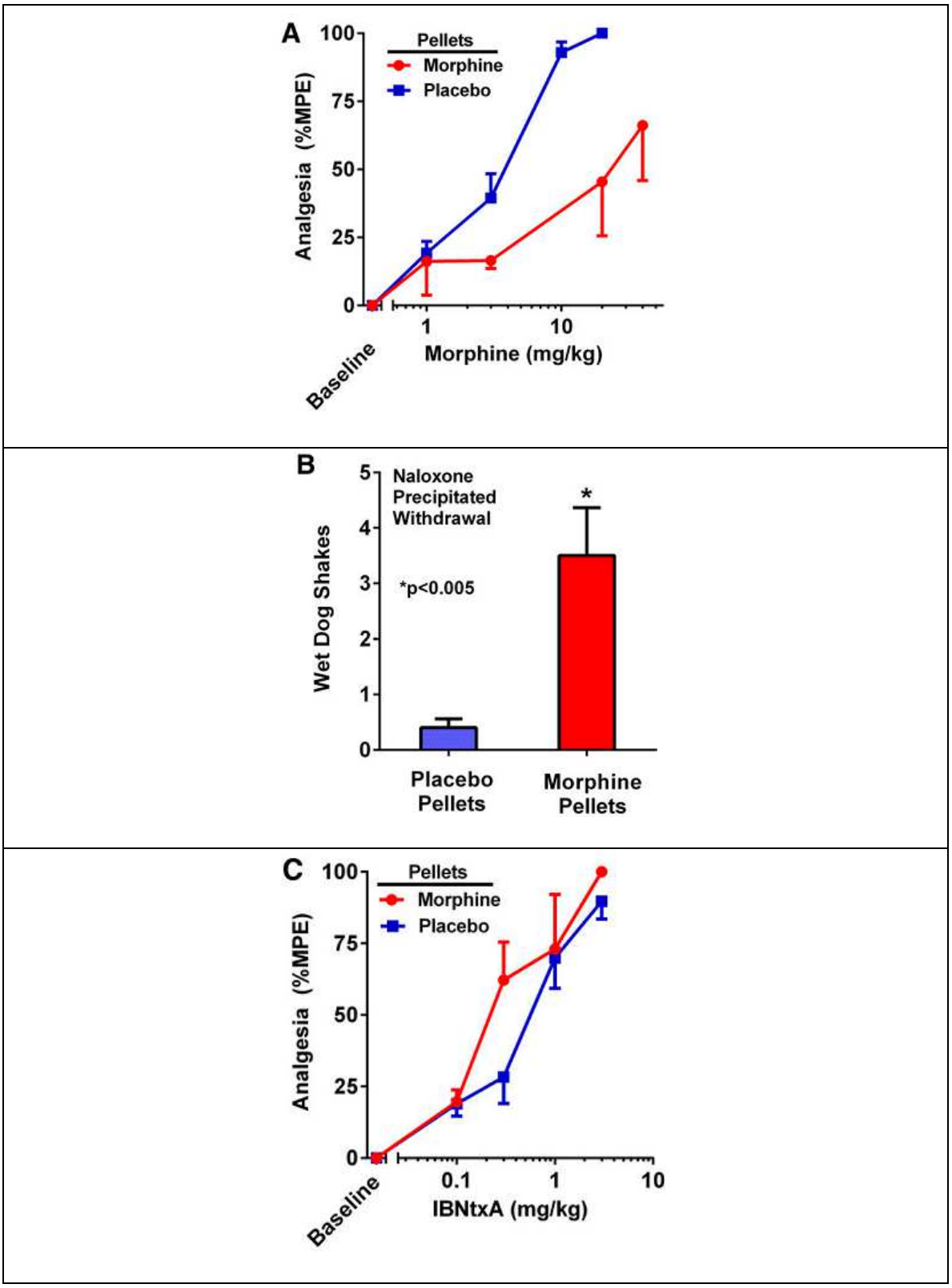
As in mice, IBNtxA was a potent analgesic in a radiant heat tail-flick assay, with an ED_{50} for subcutaneous injection of 0.89 mg/kg (Figure 8). This was approximately 2-fold less potent than was observed in mice, but still about 4-fold more potent than morphine in this assay. As in the mouse, levallorphan readily reversed the analgesia produced by IBNtxA; however, we were surprised to see that the site was much more readily reversed by naloxone, suggesting that the pharmacology of the site does differ somewhat from that observed in the mouse.

Although no opioid receptor knockout rats are currently available, cross-tolerance experiments were performed to confirm that IBNtxA analgesia was not being mediated by the traditional mu site in these animals (Figure 20). Subcutaneous implantation of 3 75mg slow-release morphine pellets produced significant tolerance in rats, confirmed by a >6-fold shift in the analgesic ED_{50} of subcutaneously injected morphine and significant naloxone precipitated withdrawal the day after cross-tolerance testing. Subcutaneous IBNtxA was actually slightly more potent in morphine pelleted animals (ED_{50} = 0.26 mg/kg) relative to animals implanted with placebo pellets (ED_{50} = 0.52 mg/kg; $p < 0.05$).

IBNtxA's effects on respiration were also assessed to determine whether IBNtxA's unique side effect profile was similar in the rat as in the mouse (Figure 21). Using pulse oximetry, oxygen saturation values were obtained following injection with saline, IBNtxA, or morphine at equipotent doses about 4-5 times their analgesic ED50s.

As expected, morphine produced a substantial decrease in blood oxygen saturation, beginning by 15 minutes post-injection and continuing until approximately 2

Figure 20: Assessment of cross-tolerance between morphine and IBNtxA in the rat.
Analgesic cross-tolerance was assessed in groups of rats, each implanted with either three morphine (n = 4) or placebo (n = 10) pellets and tested with IBNtxA 72 hours later. (A) The following day, cumulative morphine dose-response curves revealed a morphine ED_{50} [95% CI] of 20.5 mg/kg [7.7–54] in the morphine-pelleted rats compared with 3.3 mg/kg [2.7–4.0] in placebo-pelleted animals ($p < 0.001$), confirming the development of tolerance. (B) After the morphine cumulative dose-response testing, animals received a challenge dose of naloxone (1 mg/kg, s.c.) to assess physical dependence. Morphine-pelleted animals displayed a significantly greater number of wet-dog shakes, a robust withdrawal sign in rats, relative to placebo-pelleted controls ($p < 0.005$). (C) In morphine-pelleted animals, the ED50 [95% CI] for IBNtxA was 0.26 mg/kg [0.15–0.45] compared with 0.52 mg/kg [0.37–0.75] in the placebo-pelleted rats.


A
Pellets
Morphine
Placebo
Analgesia (%MPE)
100
75
50
25
0
Baseline
1
10
Morphine (mg/kg)
B
Naloxone
Precipitated
Withdrawal
*p<0.005
Wet Dog Shakes
5
4
3
2
1
0
*
Placebo
Pellets
Morphine
Pellets
C
Pellets
Morphine
Placebo
Analgesia (%MPE)
100
75
50
25
0
Baseline
0.1
1
10
IBNtxA (mg/kg)

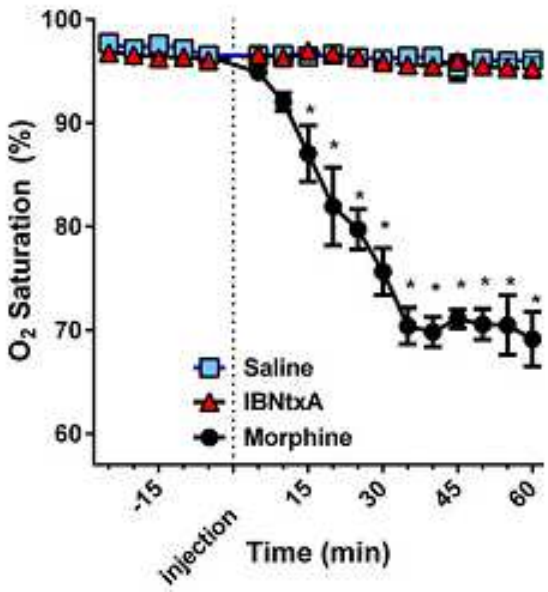


Figure 21: Respiratory depression in the rat.
Rats were randomly assigned to receive saline (n = 4), IBNtxA (4 mg/kg, n = 4), or morphine (20 mg/kg, n = 4). Using oximetry, each animal's O2 saturation was measured for 5 seconds every 5 minutes for 25 minutes before and 60 minutes after injection of the treatment. There were no differences between IBNtxA and saline at any time point, whereas morphine significantly depressed O2 saturation (* = P < 0.0001 at each time point) from 15–60 minutes as determined by Bonferroni multiple-comparison test after a two-way repeated-measures ANOVA.

hours after injection (not shown). In contrast, IBNtxA was indistinguishable from saline vehicle treated animals at any time point observed.

Using the ^{125}I-labeled form of IBNtxA, we investigated the pharmacology of the receptor binding site in rat brain membranes as well (Figure 22). As no opioid receptor knockout rat models are available, binding was performed in the presence of high concentrations (250nM each) of the selective drugs CTAP, DPDPE, and U50,488 to block binding of ^{125}IBNtxA to traditional mu, delta, and kappa receptors, respectively. Under these conditions, ^{125}IBNtxA bound a low abundance site with very high affinity (B_{max} = 15 +/- 2 fmol/mg protein; K_D = 0.22 +/- 0.02 nM). While the affinity for this site was virtually identical to the high affinity site observed in the triple knockout mouse brain (K_D = 0.16

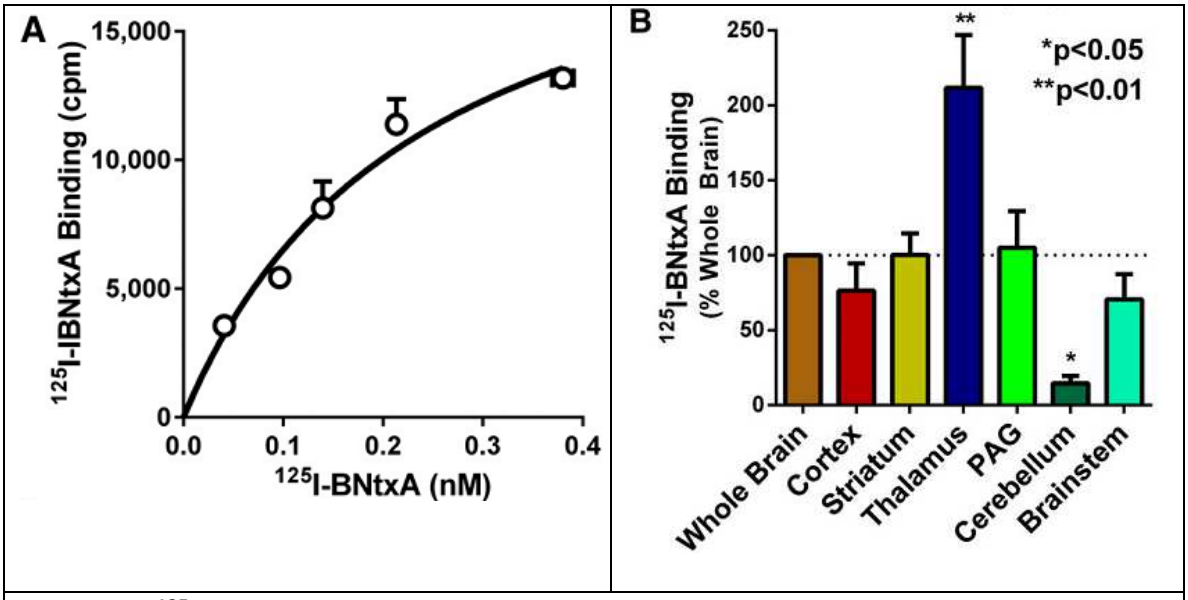


Figure 22: ^{125}IBNtxA binding in rat brain membranes.
(A) Saturation studies were performed using brain homogenates in the presence of m (CTAP), d (DPDPE), or k1 (U50,488H) blockers at 250 nM. Nonspecific binding was defined by levallorphan (1 mM). Only specific binding is reported. The assay was replicated 3 times, and the results shown are from a representative experiment. Nonlinear regression analysis using GraphPad Prism generated an average KD from the three independent experiments of 0.22 ± 0.02 nM and a Bmax of 15 ± 2 fmol/mg protein. (B) Rat brains were rapidly removed and dissected into the indicated regions, and individual homogenates were prepared. Binding in each region was determined using [^{125}I]-IBNtxA (0.1 nM) in the presence of mu, delta, and kappa$_1$ blockers (250 nM each) and normalized to binding in whole brain processed in parallel. Results are the mean ± SEM of three independent experiments. Binding differed across regions (one-way ANOVA, P = 0.0003), with the thalamus showing significantly more binding than the whole-brain control (Bonferroni multiple comparison test, P , 0.01) and the cerebellum showing significantly less binding than the whole-brain control (P , 0.05). PAG, periaqueductal gray.

+/- 0.02 nM; Majumdar, Grinnell, et al., 2011), its abundance was approximately 4-fold lower in the rat brain than in triple KO mouse brain (B_{max} = 61 +/- 2 fmol/mg) – possibly explaining the correspondingly lower analgesic ED_{50} observed in tail-flick assays described above.

The greater size of the rat brain also permitted the exploration of regional binding distribution (Figure 22B). Binding was heterogeneous across regions, with

Table 2: Competition studies of [^{125}I]IBNtxA binding in rat brain

Radioligand competition assays were performed in the presence of mu, delta, and kappa$_1$ blockers (250 nM each). Results are the mean ± S.E.M. of at least three independent determinations, each performed in triplicate. Hill slopes were determined on the binding between 15 and 85% inhibition. A number of compounds showed slopes far less than unity, consistent with binding heterogeneity - indicating that their K_i values may reflect the cumulative inhibition of multiple sites with differing affinity. In order to further address this question, several drugs were examined in greater detail (Figure 23; Table 3). The values for the triple KO mouse brain (Majumdar et al., 2011b), calf brain (Clark et al., 1989), and human neuroblastoma SK-N-BE(2)-C cell line (Standifer et al., 1994) are taken from the literature.

Drug	[^{125}I]IBNtxA			[^{3}H]NalBzoH (κ_3)	
	Rat		Triple KO Mouse Brain	Calf Striatum	Human BE(2)-C Cells
	K_i	Hill Slope	K_i	K_i	K_i
	nM		*nM*	*nM*	*nM*
μ					
Morphine	530 ± 130	−0.70 ± 0.18	>1000	32.8 ± 2.2	11.1 ± 4
Fentanyl	201 ± 92	−0.72 ± 0.23	226 ± 40		
DAMGO	>1000		>1000	8.2 ± 1.9	18.3 ± 4.5
CTAP	>1000		>1000		
Naloxone	373 ± 156	−0.83 ± 0.19	51.9 ± 1.4	8.4 ± 0.9	13.8 ± 6
δ					
Naltrindole	43.3 ± 11	−0.50[a]	26.3 ± 2.3		25.6 ± 8.7
DPDPE	>1000		>1000	>350	>1000
κ_1					
U50,488	>1000		>1000	>350	>1000
norBNI	651 ± 350	−0.53 ± 0.25	3.3 ± 1.9	103 ± 85	71 ± 9.6
Other opioid					
Levorphanol	90 ± 42	−0.55 ± 0.32	8.8 ± 2.5		
Ketocyclazocine	150 ± 34	−0.83 ± 0.18	0.04 ± 0.01	4.5 ± 0.8	
Ethylketocyclazocine	5.0 ± 1.5	−0.74 ± 0.06	0.21 ± 0.11	1.4 ± 0.5	
Nalorphine	52 ± 19	−0.63 ± 0.44	3.71 ± 1.45		
NalBzoH	30 ± 7	−0.75 ± 0.16	0.59 ± 0.15	0.9 ± 0.19	
Levallorphan	23 ± 13	−0.49 ± 0.28	0.34 ± 0.02	2.2 ± 0.56	
Buprenorphine	5.7 ± 0.7	−0.65 ± 0.24	1.8 ± 0.93		
σ_1					
(+)-Pentazocine	>1000		>1000		

DAMGO, Tyr-D-Ala-Gly-*N*-methyl-Phe-Gly-ol.
[a]This value represents a single determination.

structures such as the cortex, striatum, periacqueductal gray, and brainstem displaying moderate binding but thalamus displaying >2-fold increased abundance while cerebellum displayed >85% lower levels relative to the brain as a whole.

Finally, we examined the pharmacology of the labeled site by performing competition studies with various drugs (Table 2). As in triple knockout mouse brain binding, mu, delta, and kappa selective drugs exhibited low affinity for the site while

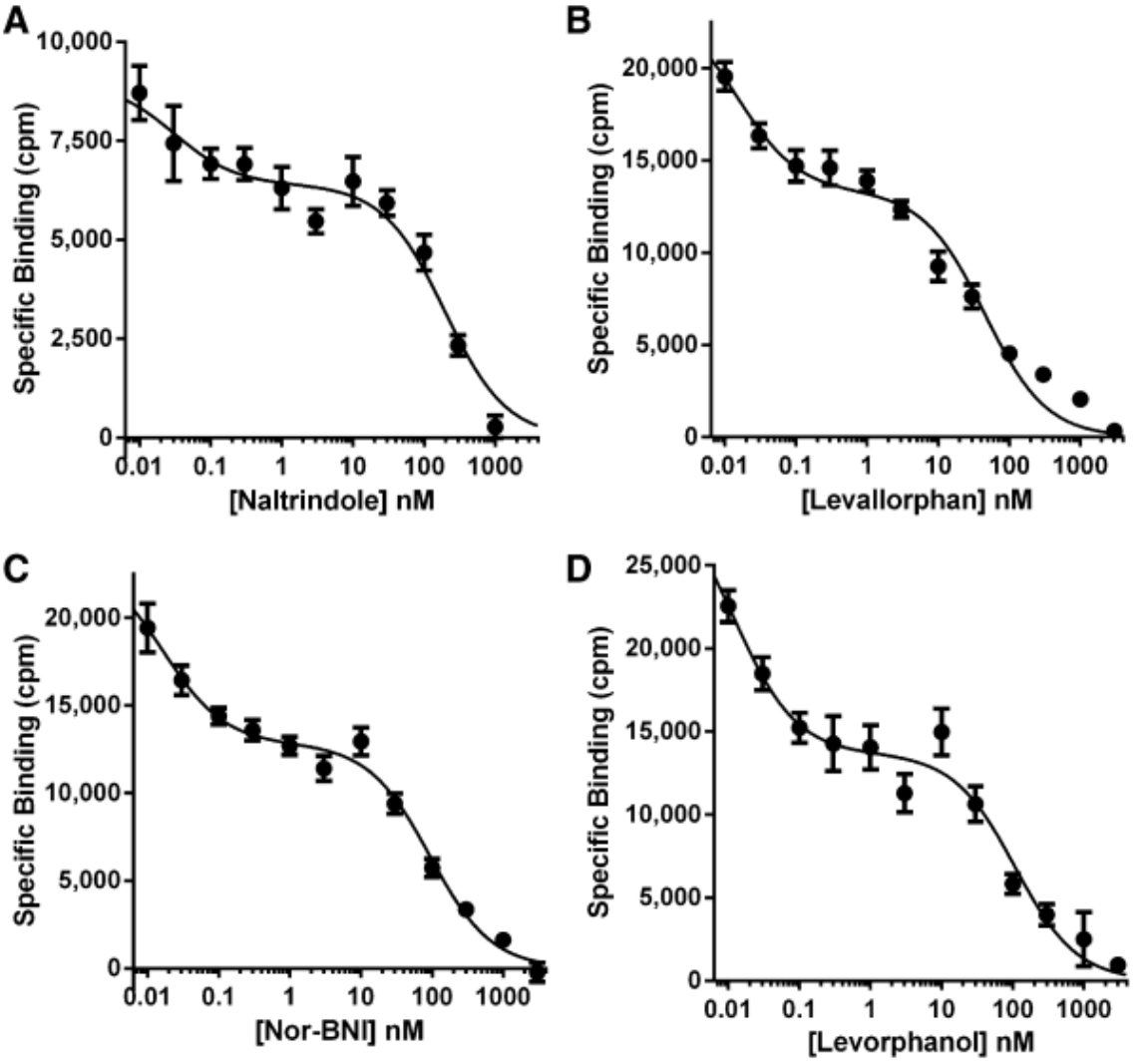


Figure 23: Detailed competition studies in rat brain membranes.
Rat brain homogenate was incubated with [^{125}I]IBNtxA (0.1 nM) with blockers at the indicated concentrations of the specified competitor. Nonspecific binding was determined using levallorphan (1 mM). Results are pooled from three independent replications perfomed in triplicate, each giving similar results, and shown as mean ± S.E.M. Each data set was fit with both a one-site and two-site model using GraphPad Prism, and the models were compared using an extra-sum-of-squares F-test. The two-site model was preferred for all four drugs tested ($P < 0.0001$ for each).

many opioids previously established as having high affinity for kappa$_3$ site as defined by [3H]-NalBzOH binding in calf striatum in the presence of EDTA (Clark et al., 1989)

competed binding with moderate to high affinity. Interestingly, buprenorphine showed very high affinity for this site in both mouse and rat brain binding.

Again, although the rat and mouse binding appeared similar overall, there were several notable differences across species, such as the significant shifts in competition curves of ketocyclazocine, norBNI, levorphanol, and levallorphan. We noticed that the hill slopes of many of these compounds tested appeared to deviate significantly from unity and therefore selected 4 of the drugs with the shallowest curves for more detailed competition studies (Figure 23). These drugs produced competition curves that were clearly biphasic, revealing high and low affinity components of their binding (Table 3) and suggesting that the target labeled in the rat by ^{125}IBNtxA may not be a single homogenous population of receptors.

Table 3: Detailed competition studies for selected opioids on rat brain ^{125}IBNtxA binding

Radioligand competition assays were performed in the presence of mu, delta, and kappa$_1$ blockers (250 nM each). Results are mean ± S.E.M. of three independent replications perfomed in triplicate, each giving similar results. Each data set was fit with both a one-site and two-site model using GraphPad Prism, and the models were compared using an extra-sum-of-squares F-test. The two-site model was preferred for all four drugs tested ($P < 0.0001$ for each).

Drug	K_i High Affinity	K_i Low Affinity	Fraction of High-Affinity Sites	Two-Site versus One-Site Model *P* Value
		nM		
Naltrindole	0.06 ± 0.03	321 ± 160	27 ± 1%	<0.001
Levallorphan	0.12 ± 0.03	119 ± 26	35 ± 5%	<0.001
norBNI	0.08 ± 0.01	184 ± 23	34 ± 7%	<0.001
Levorphanol	0.07 ± 0.01	219 ± 44	42 ± 8%	<0.001

Discussion

The quest for the ideal analgesic has consumed decades of research and produced thousands of new opioid compounds. Despite substantial advances in our understanding of opioid receptor pharmacology, the mainstays of pain management –

analgesics targeting the mu opioid receptor such as morphine, fentanyl, and oxycodone – still exhibit the same problematic side effect profile of tolerance, dependence, constipation, respiratory depression, and euphoria resulting in abuse and addiction. The history of opioid pharmacology has been to discover and exploit receptor heterogeneity to maximize desired effects while minimizing off-target side effects, splitting "opioid receptors" first into mu, delta, and kappa subtypes, and then subtypes of each of these receptors defined pharmacologically as reviewed in Chapter 1. Recent efforts have focused on agonist bias, whereby drugs may preferentially activate different signaling pathways which mediate different effects of drugs (Bohn et al., 2000; Raehal et al., 2005). Other studies have revealed the existence of homo- and heteromeric signaling complexes comprised of multiple GPCRs, which exhibit unique properties from their component receptors and may be specifically targeted by drugs (Gomes et al., 2004, 2013; Jordan & Devi, 1999; Y-X Pan et al., 2002; Yekkirala et al., 2011; Yekkirala, Kalyuzhny, & Portoghese, 2010).

Our approach has focused on reconciling the pharmacologically defined opioid receptor subtypes with the extensive alternative splicing observed for the mu opioid receptor gene. Indeed, this focus on splice variants is complementary with research on ligand bias and heterooligomerization as different splice variants are likely to interact with different complement of proteins to mediate their effects, and their expression is known to differ in a regionally and cell-type specific manner (Abbadie, Pan, Drake, & Pasternak, 2000; Abbadie et al., 2004). Although truncated 6TM mu receptor variants were described over a decade ago, their deviation from the canonical 7TM GPCR structure and inability to bind ligands when expressed heterologously called their functional relevance into question (Ying-Xian Pan et al., 2001). The generation of an

MOR exon 11-knockout animal has definitively established a role for these truncated receptors in opioid pharmacology for drugs both new and old.

Here, a novel opioid pain reliever, IBNtxA, was shown to exhibit powerful analgesia with a vastly superior side effect profile. In both mice and rats, IBNtxA was several-fold more potent than morphine in the radiant heat tail-flick assay, and other studies have since demonstrated its broad analgesic efficacy in other models of pain (Wieskopf et al., 2014). More importantly, however, IBNtxA showed a superior safety profile, producing no respiratory depression in either mice or rats. In both rodent species, IBNtxA was not cross-tolerant with morphine and also failed to precipitate withdrawal in tolerant animals. In mice, IBNtxA continued to show a limited amount of constipation, but this off-target effect may be avoided by more selective compounds targeting the site labeled by this drug. Importantly, its benign character in a mouse conditioned place preference assay suggests low liability for diversion and abuse of the drug although future studies will be needed to confirm these findings in other species and models of drug reward and reinforcement.

[^{125}I]IBNtxA binding in the rat brain revealed the regional distribution of the likely target producing the drug's analgesic effects, with highest levels observed in the thalamus – a region critically involved in pain pathways (Monconduit, Bourgeais, Bernard, Le Bars, & Villanueva, 1999; A. Pert & Yaksh, 1974; Pozza et al., 2010; Zubieta et al., 2001). Competition studies in mouse and rat showed low affinity of many selective mu, delta, and kappa compounds for the site while other compounds such as naloxone benzoylhydrazone, the prototypical "kappa$_3$" drug, exhibited higher affinity, suggesting some overlap of this target with the pharmacologically defined kappa$_3$ site identified decades ago (Clark et al., 1989). Indeed, naloxone benzoylhydrazone analgesia is absent in MOR exon 11-knockout animals, consistent with this identity (Majumdar,

Grinnell, et al., 2011). However, the substantially lower B_{max} of [^{125}I]IBNtxA versus [^{3}H]-naloxone benzoylhydrazone in mouse brain suggests that [^{125}I]IBNtxA is labeling only a subpopulation of these sites.

Despite the widespread similarity between findings in mice and rats, binding was notably different between species. Although the affinity for the target in the presence of selective mu, delta, and kappa blockers did not significantly differ between species, [^{125}I]IBNtxA binding was much lower in the rat brain (B_{max} = 15 fmol/mg protein) relative to the mouse (B_{max} = 60 fmol/mg protein in triple knockout brain). Additionally, although trends in competition studies were similar between species, most drugs with high affinity against [^{125}I]IBNtxA in triple knockout mouse brain competed [^{125}I]IBNtxA binding in rat brain at least an order of magnitude less potently. This may be explained in part by the heterogeneity observed in the shallow hill slopes and more detailed competition studies which revealed the presence of both very high and low affinity components competed by the drugs examined; thus, the "apparent K_i" values reported must be interpreted with caution.

It is unclear what these two sites represent genetically, although the addition of 250nM mu, delta, and kappa blockers in these assays argues against these traditional opioid receptors. Although mu opioid receptor splicing patterns are broadly conserved across mouse, rat, and human genomes, the rat MOR-1 splicing produces two isoforms of the truncated MOR-1G isoform implicated in IBNtxA analgesia by rescue(Lu, Xu, Pasternak, and Pan, accepted manuscript). These isoforms, rMOR-1G1 and rMOR-1G2, are homologous with the human isoforms, hMOR-1G1 and hMOR-1G2 which were isolated from human brain tissue(Xu, Xu, Hurd, Pasternak, & Pan, 2009). These splice variants are also expressed in SK-N-BE(2)C cells, a human neuroblastoma cell line which also exhibits [^{125}I]IBNtxA and [^{3}H]-Naloxone benzoylhydrazone binding (Mathis,

Mandyam, Altememi, Pasternak, & Standifer, 2001). However, it is unlikely that these two isoforms represent the high and low affinity components of [^{125}I]IBNtxA binding in rat brain as the 6TM protein products of these genes differ by only 7 amino acids (MGSGPML) present at the N-terminus of rMOR-1G2. Another tantalizing possibility is that the 6TM isoform forms complexes with multiple other proteins, producing multiple active signaling complexes – targets for future attempts at exploiting opioid receptor multiplicity toward the ultimate goal of the powerful pain reliever lacking side effects.

Chapter 4: Receptor mechanisms of an unusual opioid, buprenorphine: Involvement of MOR Exon 11-associated Splice Variants

Introduction

Buprenorphine was synthesized nearly 50 years ago as one of a large series of semi-synthetic opioids referred to as the "Bentley Compounds," – or, more technically, orvinols and thevinols. While at the University of Aberdeen in the early 1960s, pioneering opioid organic chemist Kenneth Bentley had begun experimenting with Diels-Alder adducts of thebaine and oripavine. Shortly thereafter, Bentley was hired by pharmaceutical research and manufacturing firm McFarlan Smith, assembling a team of medicinal chemists in a joint research program with British consumer product manufacturer Rickett & Colman[3] which would famously synthesize and test numerous semi-synthetic opioid derivatives over the next decade.

Thebaine and oripavine are morphinan alkaloids occurring naturally in opium and poppy straw although at lower concentrations than morphine and codeine, and are only moderately potent analgesics on their own. However, using the C-ring of the these alkaloids as a diene in a Diels-Alder [4 + 2] addition, a bridged ring system is formed by reaction with various dienophiles such as methyl vinyl ketone, leaving behind a new ketone which is readily diversified by alkylation with numerous Grignard reagents. Subsequent substitutions at the 3-hydroxy and 17-N-methyl positions, and saturations of the 7,8-double bond further resulted in thousands new semi-synthetic opioids which

[3] Lewis (1999) recollects the peculiar circumstances leading Rickett & Colman, a company whose business primarily involved producing grocery products, to fund a major research campaign resulting in the production of thousands of novel opioids - some of which were thousands of times more potent than morphine. In fact, their initial goal was simply to replace codeine in one of the company's over-the-counter formulations of aspirin.

were tested for retention of morphine's analgesic effects but superior side effect profiles by Bentley's research group.

Early success was achieved with compound M99, named etorphine, which was several thousand-fold more potent than morphine in various rodent models of thermal and mechanical pain (Bentley & Hardy, 1963; Blane, Boura, Fitzgerald, & Lister, 1967; Lister, 1964). However, etorphine suffered essentially the same side effect profile as existing mu opioids, and reports that minute quantities had been used to immobilize large game such as elephants or rhinocerii quickly caught media attention and inflamed the imaginations of regulators fearing a new drug addiction epidemic of "ultra-potent heroin." Thus, although Janssen's discovery of the similarly super potent analgesic fentanyl resulted in enormous commercial success and widespread use today, the World Health Organization deemed etorphine too dangerous for humans and it was added to the most restrictive list, Schedule 4. Its use is still limited to large animal veterinary practice worldwide, although the even more potent 7,8-unsaturated derivative dihydroetorphine is used in China as an analgesic and in opioid maintenance therapy for the treatment of opioid dependence (Ohmori & Morimoto, 2002; Qin, 1996).

Later efforts focused on N-cyclopropylmethyl orvinol derivatives, as this moiety was known to produce antagonist effects when substituted on the morphinan pharmacophore. Compounds M278 and M285 appeared promising in rodents as potent analgesics which did not produce a morphine withdrawal syndrome, but early human tests revealed psychotomimetic and dysphoric effects which we now know result from their agonist activity at kappa receptors. Another candidate, M5205, showed weak activity in humans, some dysphoria, and a morphine-like withdrawal effects in rhesus monkeys. The failure of the group to produce a clinically viable product began to

concern Rickett & Colman and the group decided to reexamine some of the orvinols which had previously been rejected on the basis of rat tail-pressure screening, particularly several which exhibited bell-shaped curves in the rat warm water tail-withdrawal test (Cowan, Lewis, & Macfarlane, 1977; recounted in Lewis, 1999). These drugs were found to act as partial agonists, antagonizing morphine effects but also capable of producing analgesia when administered on their own, a property the group hypothesized would translate into lower abuse potential. Tests of withdrawal syndromes in patas monkeys revealed that one of these drugs, M6029, did not display either spontaneous or precipitated withdrawal symptoms (Cowan, Lewis, et al., 1977). The group selected this drug - later named buprenorphine - to be its final candidate for human trials. Several of the group's chief scientists including John Lewis and Alan Cowan were among the first volunteers to receive the drug intravenously, finding it to be generally safe and well-tolerated after acute administration, although Lewis suffered nausea and vomiting – a notable side effect of the drug shared with other mu analgesics. Oral formulations demonstrated low bioavailability but a sublingual formulation was successfully developed and carried into clinical trials in post-operative pain.

Alan Cowan studied the pharmacology of buprenorphine in animals extensively (Cowan, Doxey, & Harry, 1977; Cowan, Lewis, et al., 1977). In rodents, buprenorphine is a potent analgesic with an analgesic ED_{50} >25-fold lower than that of morphine and a considerably longer duration of action. As previously noted, it produces a bell-shaped curve in thermal pain models such as warm-water tail-withdrawal and tail flick, with doses up to 3 mg/kg showing increasing levels of analgesia and a subsequent descending arm observed at higher doses. Tolerance developed to the drug's analgesic effects, albeit more slowly than to morphine, and animals showed cross-tolerance

between morphine and buprenorphine. In rodents, low doses of buprenorphine can antagonize morphine analgesia, and it precipitates a partial withdrawal syndrome in morphine-dependent mice and monkeys but not rats. Importantly, as noted previously, monkeys made tolerant to buprenorphine fail to demonstrate a significant withdrawal syndrome.

Buprenorphine also showed a superior side effect profile to morphine, particularly with respect to respiratory effects. Although some respiratory depression was observed in rats, the drug showed a ceiling effect with increasing doses, and the maximal effect on PCO_2 was just half that produced by morphine. Accordingly, the therapeutic window between analgesic ED50 and LD50 was dramatically improved over that of morphine. Furthermore, a bell-shaped curve was seen for inhibition of gastrointestinal transit, with doses up to 0.3mg/kg decreasing transit and doses above this amount producing decreasing effects to the levels observed in saline controls. The effects observed in animals largely mirrored those subsequently observed in humans (reviewed in Pergolizzi et al., 2010). In particular, although *in vitro* assays such as stimulation of [^{35}S]-GTPγS binding often show buprenorphine as a partial agonist at mu opioid receptors, it appears to be fully efficacious using analgesia in humans as an endpoint (Dahan et al., 2006; Raffa et al., 2014); indeed, Pergolizzi and Raffa, et al. go to great lengths to clarify that it is impossible to predict based solely on *in vitro* functional assays whether a drug will behave as a full or partial agonist *in vivo* in a given tissue, endpoint, and context (such as dependent vs. nondependent patients). Critically, using respiratory depression in humans as an endpoint, buprenorphine truly does manifest the properties of a partial agonist, displaying a ceiling effect with maximal inhibition significantly lower than other agonists such as morphine and fentanyl (Dahan et al., 2005, 2006). As in rodent models, this is manifest in a wider therapeutic window

between effective dose and toxic/lethal dose, providing an increased margin of safety over other more commonly used opioids in clinical practice (Pergolizzi et al., 2010; Yassen, Olofsen, Kan, Dahan, & Danhof, 2008). One caveat, however, is that reversal of buprenorphine induced respiratory depression requires higher doses of naloxone than would be required for other clinically used opioids, and also shows a bell shaped curve hinting at the involvement of multiple receptor systems (Dorp et al., 2006).

At first glance, buprenorphine shows many similarities with IBNtxA as a powerful pain reliever with a superior side effect profile to other opioid analgesics. However, despite these improvements, buprenorphine still produces clinically relevant constipation, and produces euphoria and drug-liking, especially in non-opioid dependent users (Duke, Correia, Walsh, Bigelow, & Strain, 2010; Jones, Madera, & Comer, 2014; Middleton, Nuzzo, Lofwall, Moody, & Walsh, 2011). Notably, abuse is seen at lower levels relative to conventional opioids - perhaps due to the potential for precipitation of withdrawal, either by buprenorphine itself or naltrexone also present in the more widely used sublingual formulation, Subutex® (Alho, Sinclair, Vuori, & Holopainen, 2007; Cicero & Inciardi, 2005). Intriguingly, while buprenorphine analgesia is lost in knockout animals lacking exon-2 associated variants of the mu opioid receptor, potentiation of analgesia by an ORL_1 antagonist, J-113397 and in ORL_1 knockout animals points to as yet unappreciated receptor mechanisms mediating buprenorphine's effects (Lutfy et al., 2003). Other studies have suggested multiple sites of action for buprenorphine analgesia, with spinal analgesia blocked by naloxone but supraspinal analgesia appearing naloxone-insensitive (Ding & Raffa, 2009). Additionally, buprenorphine shows analgesic cross-tolerance with naloxone benzoylhydrazone, the prototypical $kappa_3$ agonist (Pick, Peter, Schreiber, & Weizman, 1997), and competes [^{125}I]-IBNtxA binding with high affinity in binding assays in triple opioid knockout mouse brain, as well

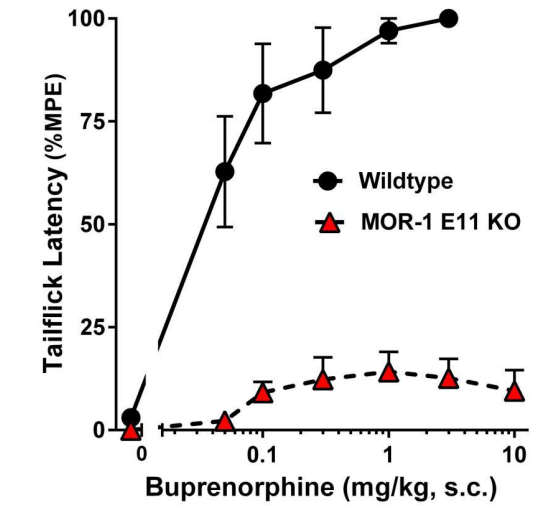
as mouse or rat brain in the presence of mu, delta, and kappa blockers (Grinnell et al., 2014; Majumdar, Grinnell, et al., 2011). We therefore sought to examine the role of exon 11-associated and exon 1-associated MOR splice variants in buprenorphine's effects.

Results

As discussed previously, some mu opioids such as morphine and methadone, lose all analgesia in an MOR exon 1 knockout animal, while drugs like M6G and heroin retain full efficacy with only slight loss in potency (A. G. Schuller et al., 1999). MOR Exon 11 knockout animals display a total loss of analgesia to IBNtxA, and a decrease in potency is observed to fentanyl, M6G and heroin, while morphine and methadone analgesia are unaffected (Majumdar, Grinnell, et al., 2011; Ying-Xian Pan et al., 2009).

In tail flick tests, buprenorphine showed a unique pharmacological profile, displaying sensitivity to loss of both exon 11- and exon 1-associated MOR variants (Figure 24). In wildtype C57 animals buprenorphine was extremely potent, with an analgesic ED_{50} of 0.028 mg/kg, similar to previous reports. Like IBNtxA, buprenorphine analgesia appeared essentially inactive in exon 11 knockout animals even at doses >300-fold greater than the wildtype ED_{50} (Figure 24A). The picture was more complicated for exon 1 knockout animals, where analgesia was observed, plateauing at approximately 40%. Furthermore, the ED_{50} for this partial response did not differ significantly from the ED_{50} in wildtype 129/S6 controls. Clearly, multiple receptor mechanisms contribute to buprenorphine's analgesic effects, with full response requiring both exon 1- and exon 11-associated variants.

Binding studies have clearly shown that buprenorphine binds to traditional mu opioid receptors with high affinity (P Huang, Kehner, Cowan, & Liu-Chen, 2001). We therefore attempted to determine, whether buprenorphine was still capable of



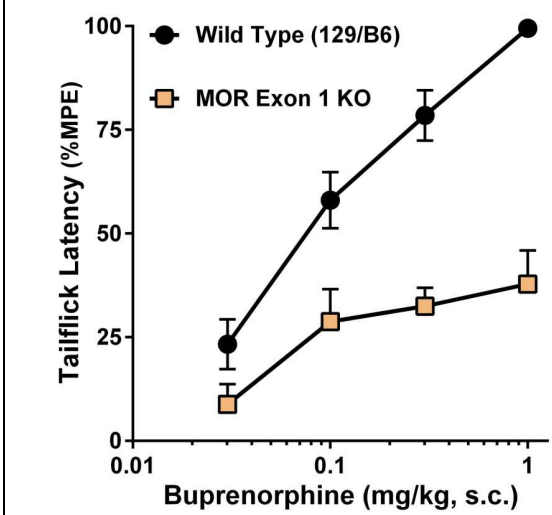


Figure 24: Buprenorphine analgesia in MOR-1 exon 11 and exon 1 knockout mice.
Mice were injected with escalating doses of buprenorphine and their tail flick latencies tested 30 minutes later to generate dose-response curves. Results are pooled from 2 independent experiments giving similar results and are expressed as mean ± SEM. (A) Buprenorphine analgesia is lost in exon 11 mice. Buprenorphine was a potent analgesic in wildtype C57BL/6 mice (n = 9), with an ED_{50} value 0.028 mg/kg (0.015 – 0.051), while in MOR exon 11-knockout animals (n = 20) it showed only a slight elevation over baseline latency. There was a significant difference between curves ($p<0.0001$, Extra sum-of-squares F test). (B) Buprenorphine analgesia is significantly reduced in MOR exon 1 and triple KO mice. Buprenorphine was also a potent analgesic in wildtype 129S6 mice (n = 20-24) with an ED_{50} value of 0.079 mg/kg (0.048 – 0.13). In MOR exon 1 KO (n = 12) and triple KO (n = 7-11) mice, buprenorphine appeared to be a partial agonist with a maximal effect of approximately 40% of MPE. For these curves, the ED_{50} values were 0.064 mg/kg (0.011 – 0.37) for the MOR exon 1 KO and 0.067 mg/kg (0.017 – 0.26) for the triple KO. There was no significant difference between dose response curves for MOR exon 1 and triple KO animals, and no significant difference for ED_{50} values between all 3 genotypes, but a significant difference was observed for maximal effect ($p<0.0001$; Extra sum-of-squares F test).

interacting with full length mu receptors which are still expressed in MOR exon 11 knockout animals despite loss of analgesic effects (Ying-Xian Pan et al., 2009). Indeed, buprenorphine reversed analgesia produced by morphine pretreatment, suggesting that the drug still binds mu receptors and appears to compete with morphine for receptor

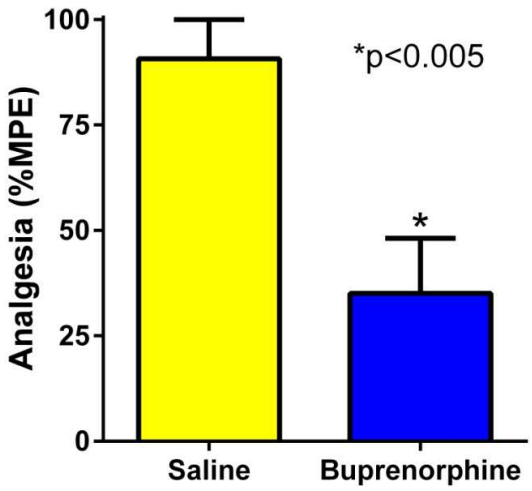


Figure 25: Buprenorphine reversal of morphine analgesia in MOR Exon 11 knockout animals.
Animals were given morphine (10 mg/kg) and their tail flick latencies tested 30 minutes later at peak effect. Animals were assessed quantally as a doubling or greater of baseline latency, and analgesic animals were randomly assigned to receive either saline or buprenorphine (10 mg/kg). 15 minutes after receiving the second injection, animals were retested in the tail flick test. Buprenorphine significantly decreased morphine analgesia relative to saline control (*, $p<0.003$, 2-tailed t-test). Results are pooled from 2 independent experiments with similar results and are expressed as mean ± SEM.

occupancy, but curiously fails to produce analgesia in the absence of exon 11-associated MOR variants.

Buprenorphine binds opioid receptors relatively nonselectively. We next sought to determine whether other DOR, KOR, or ORL1 receptors contributed to the curious pharmacological profile observed in Exon 1 and Exon 11 knockout animals. However, there was no difference between dose response curves between any individual KOR or ORL_1 knockout and wildtype 129S6 controls, and the triple opioid receptor knockout generated by crossing MOR exon 1 knockout animals with DOR-1 and KOR-1 knockout animals appeared no different from MOR exon 1 knockout alone, displaying analgesia which plateaued at approximately 40% response (%MPE) with an ED_{50} for this partial response indistinguishable from the MOR exon 1 knockout and wildtype ED_{50}. Taken

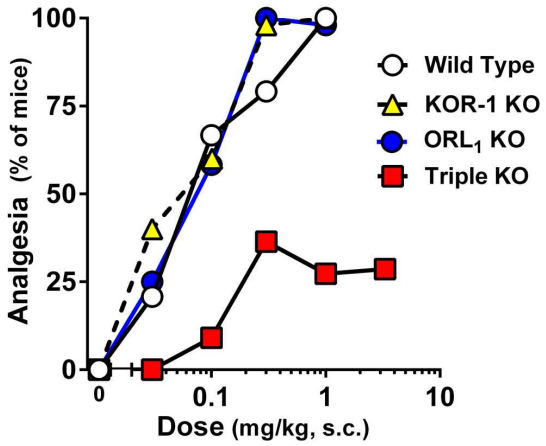


Figure 26: Buprenorphine analgesia in DOR, KOR, ORL-1, and triple opioid receptor knockout mice

Mice were injected with escalating doses of buprenorphine and their tail flick latencies tested 30 minutes later to generate dose-response curves. DOR-1, KOR-1, and ORL-1 knockout animals did display any difference from wildtype 129S6 controls, while a triple opioid receptor knockout animal lacking MOR exon 1 reached a plateau at 30% of animals displaying at least a doubling of baseline latency, which did not differ from MOR exon 1 knockout alone (Extra sum-of-squares F test).

together, these studies suggest that buprenorphine analgesia is mediated exclusively via mu opioid receptors, consistent with previous findings in MOR exon 2 knockout animals (Lutfy et al., 2003).

The most definitive demonstration of the involvement of exon 11-associated MOR splice variants came via a rescue experiment, where a lentiviral construct was used to restore expression of mMOR-1G, one of the truncated 6TM receptors lost in exon 11 knockout animals. In exon 1/exon 11 double knockout animals which express no remaining mu receptor splice variants, lentiviral mMOR-1G was able to rescue IBNtxA but not buprenorphine analgesia, consistent with findings herein that buprenorphine requires both exon 11 and exon 1 variants for full activity (Lu et al., 2015). However, when given either i.c.v. or i.t. to exon 11 knockout animals, lentiviral mMOR-1G

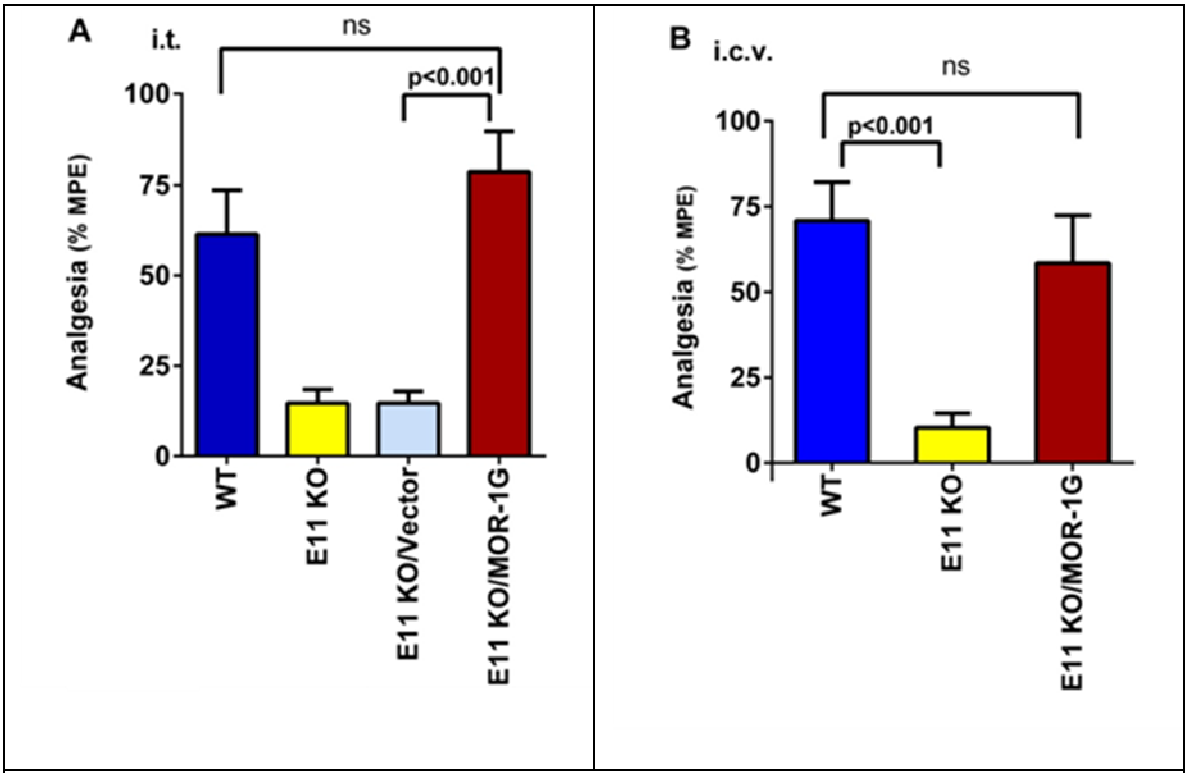
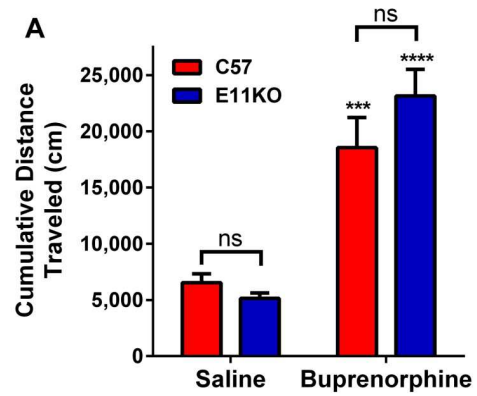


Figure 27: Rescue of buprenorphine analgesia by lentiviral delivery of MOR-1G in MOR E11 Knockout.

A lentivirus expressing EGFP and mMOR-1G or EGFP alone (vector) was injected and tested for analgesia by buprenorphine given systemically (1 mg/kg, s.c.) at least 5 weeks later. (A) Intrathecal lentivirus: Buprenorphine analgesia was significantly lowered in the E11 KO relative to wildtype controls, consistent with results in dose-response studies (Figure 24A). There was no effect of vector lentivirus treatment in knockout animals, but mMOR-1G lentivirus rescued buprenorphine analgesia relative to both E11 KO and E11 KO animals treated with vector lentirus. There was no significant difference between analgesia in wildtype mice vs E11 KO mice treated with mMOR-1G lentivirus. (Bonferroni multiple comparison post-hoc test following one-way ANOVA). (B) Intracerebroventricular lentivirus: Buprenorphine analgesia was significantly lowered in the E11 KO relative to wildtype controls, consistent with results in dose-response studies (Figure 24A). There was no significant difference between analgesia in wildtype mice vs E11 KO treated with mMOR-1G lentivirus. (Bonferroni multiple comparison post-hoc test following one-way ANOVA).

rescued systemic buprenorphine analgesia to levels indistinguishable from wildtype controls, strongly implicating 6TM variants in the analgesic effects of buprenorphine.



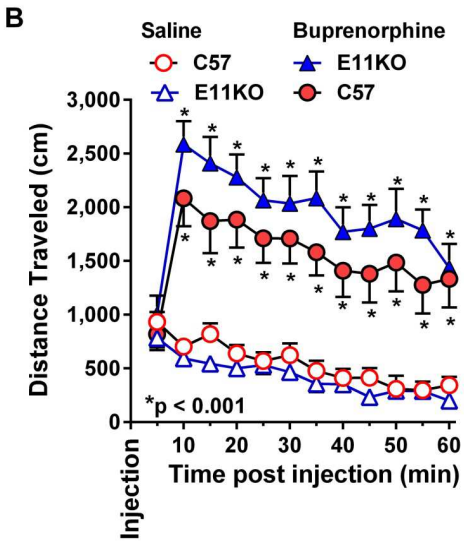


Figure 28: Effect of buprenorphine on locomotor behavior in MOR Exon 11 KO mice. Animals (n = 7 for each group) were injected with saline or buprenorphine (3 mg/kg s.c.), and their locomotor behavior recorded in an open field activity chamber for 60 minutes post-injection. Results are mean ± SEM. (A) Total distance traveled during the 60 min session was significantly increased by buprenorphine injection in both genotypes relative to saline vehicle control ($p < 0.0001$ for a main effect of drug, 2-way repeated measures ANOVA; $p<0.001$ for C57 and $p<0.0001$ for E11KO, post-hoc Tukey's multiple comparisons test); however, there was no difference between genotypes for either saline or buprenorphine injection ($p = 0.39$ for a main effect of genotype, $p = 0.12$ for drug x genotype interaction; $p = 0.95$ for saline comparison, $p = 0.31$ for buprenorphine comparison). (B) Time course of locomotor activity, 5 minute intervals. Within 10 minutes post-injection, buprenorphine significantly increased locomotor behavior in both MOR exon 11 knockout animals and wildtype C57 controls ($p < 0.001$ for each point minutes 10-60; post-hoc Tukey's multiple comparisons test). There was no significant difference between genotypes at any time point for either saline or buprenorphine condition.

We next assessed the role of exon 11-associated splice variants with buprenorphine's side effects to determine whether the same targets mediate both the desirable and undesireable effects of the drug. First, we looked at stimulation of locomotor activity. Although not a direct measure of reward, opioid-induced locomotor stimulation is mediated by disinhibition of dopamine release in the mesolimbic

dopamine pathway, the common pathway shared by drugs of abuse (Johnson & North, 1992; Joyce & Iversen, 1979; Urs, Daigle, & Caron, 2011).

Administration of a 3 mg/kg dose of buprenorphine produced a robust increase in locomotor activity in MOR Exon 11 knockout animals despite the fact that this dose did not produce a significant analgesic response in the tail flick assay. There was also no significant difference between MOR exon 11 KO animals either in response to saline or to buprenorphine, and no difference in time course to either treatment.

Next, we looked at inhibition of gastrointestinal transit, a model of constipation which can be a dose-limiting side effect of opioid analgesics including buprenorphine. In this assay, pretreatment with a moderate dose of 0.3 mg/kg buprenorphine produced robust inhibition of transit relative to either C57 or Exon 11 KO animals pretreated with saline. GI transit appeared higher in Exon 11 knockout animals, although this didn't reach significance, raising the question of whether Exon 11-associated variants might actually be involved tonically in the modulation of gut motility. Further studies with greater statistical power will be required to answer this question. Taken together, these studies suggest that the receptor mechanisms responsible for disinhibition of mesolimbic dopamine release and inhibition of gastrointestinal transit are not dependent on exon 11-associated variants and that these effects are dissociable from the analgesic actions of the drug.

In vitro functional assays were largely consistent with previous reports on the pharmacology of buprenorphine (Figure 30). In our hands, however, buprenorphine behaved as a full agonist in CHO cells stably expressing mMOR-1 - contrary to its widespread characterization as a partial agonist in this assay (Romero et al., 1999; Selley, Sim, Xiao, Liu, & Childers, 1997). Differences between our results and those

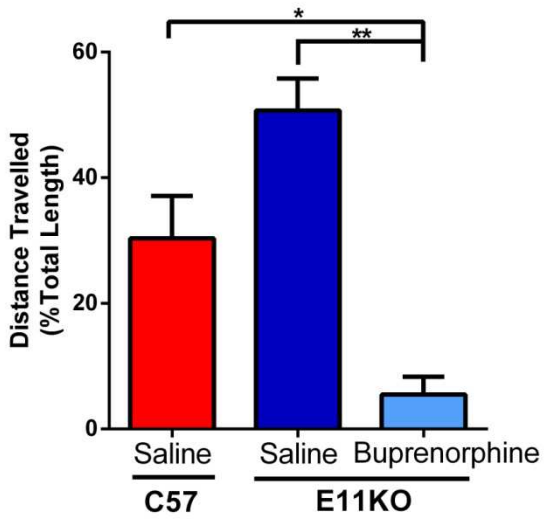
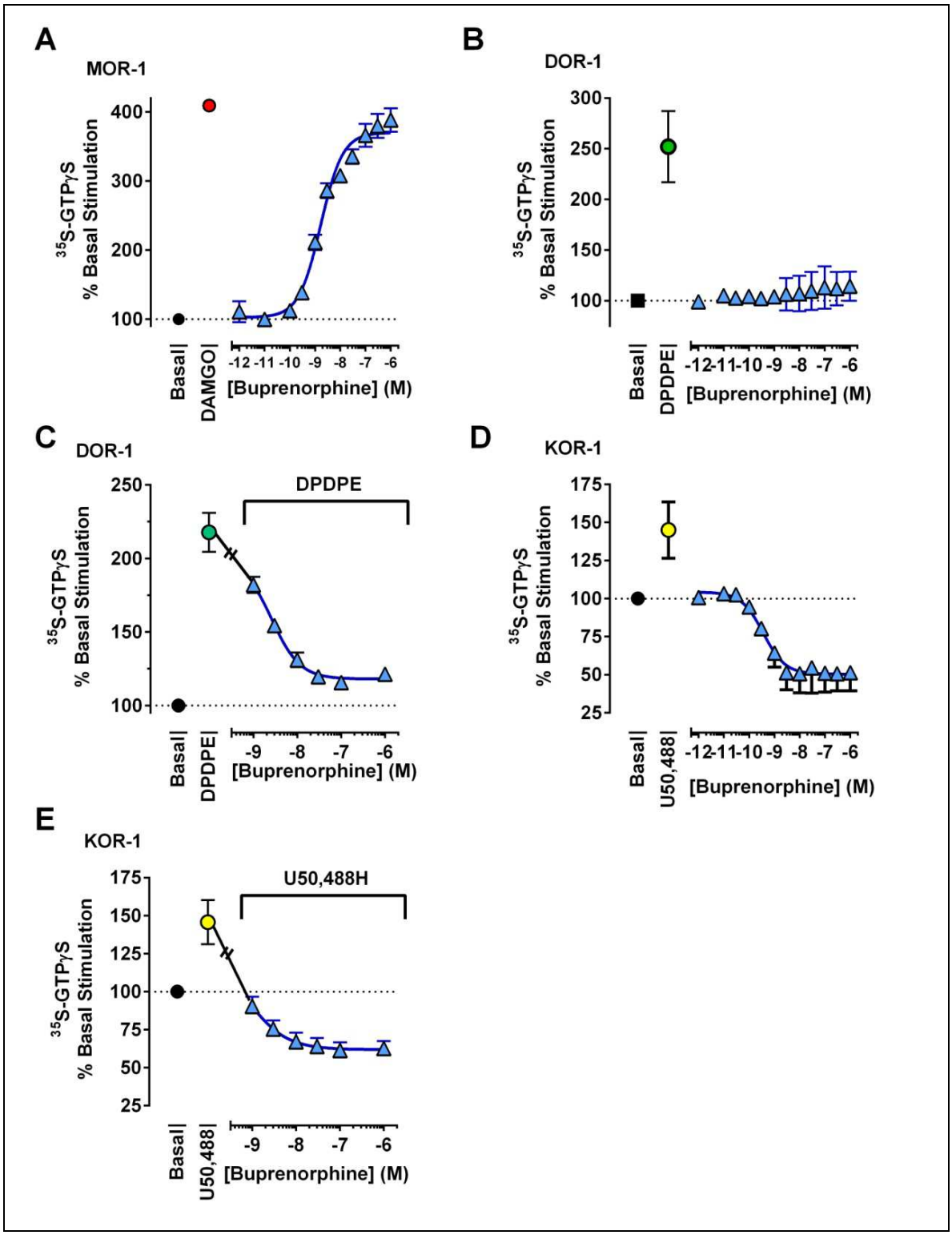


Figure 29: Effect of Buprenorphine on GI transit in MOR Exon 11 Knockout animals. Exon 11 knockout mice (n = 7 per group) or wildtype C57 controls (n = 6) were injected with either saline or buprenorphine (0.3 mg/kg s.c.) and 10 minutes later given a charcoal meal by gavage. 30 minutes after administration of the charcoal meal, animals were sacrificed by cervical dislocation and the distance travelled by the charcoal meal was measured as a fraction of the total distance from the pyloric sphincter to the cecum. Buprenorphine significantly decreased gastrointestinal motility relative to both saline-treated Exon 11 knockout controls ($p < 0.0001$, post-hoc Bonferroni multiple comparisons test following 1-way ANOVA) and saline-treated wildtype C57 controls ($p < 0.01$). Results are mean ± SEM.

reported in the literature can be readily explained by the use of different clones of CHO-MOR stable cells generated in each lab. Differences in receptor expression levels and G-protein stoichiometry will have profound effects on the maximal stimulation obtained in response to both experimental drugs as well as the reference full agonist (DAMGO). Specifically, nonlinearity due to ceiling effects may result in partial agonists appearing as full agonists. Alone, buprenorphine acted as a relatively neutral antagonist and inverse agonist in mDOR and mKOR expressing cells, respectively. Consistent with this characterization, buprenorphine potently reversed stimulation produced by DPDPE or U50,488.It was also clear that functional assays in heterologously expressing cell lines did not match the effects observed in brain tissue. Buprenorphine produced virtually no

Figure 30: Effect of buprenorphine on ^{35}S-GTPγS binding in cell lines.
35S-GTPγS binding assays were performed with membrane homogenates from CHO cells stably expressing the indicated opioid receptor. Results are pooled from 3 independent replications and are expressed as mean ± SEM. (A) At MOR-1, buprenorphine acted as a full agonist relative to 1uM DAMGO control with an EC_{50} of 1.8nM (1.3, 2.3). (B) At DOR-1, buprenorphine produced little or no stimulation of 35S-GTPγS binding above basal levels, and (C) potently antagonized 1uM DPDPE stimulation. (D) At KOR-1, buprenorphine behaved as an inverse agonist, potently reducing 35S-GTPγS binding more than 50% below basal levels (IC_{50} = 0.36 nM [0.11. 1.4]) and (E) reversed stimulation produced by 1uM U50,488 (IC_{50} = 0.69 nM) with higher concentrations further reducing binding below basal levels.


A
MOR-1
^{35}S-GTPγS
% Basal Stimulation
400
300
200
100
Basal
DAMGO
-12 -11 -10 -9 -8 -7 -6
[Buprenorphine] (M)
B
DOR-1
^{35}S-GTPγS
% Basal Stimulation
300
250
200
150
100
Basal
DPDPE
-12 -11 -10 -9 -8 -7 -6
[Buprenorphine] (M)
C
DOR-1
DPDPE
^{35}S-GTPγS
% Basal Stimulation
250
200
150
100
Basal
DPDPE
-9 -8 -7 -6
[Buprenorphine] (M)
D
KOR-1
^{35}S-GTPγS
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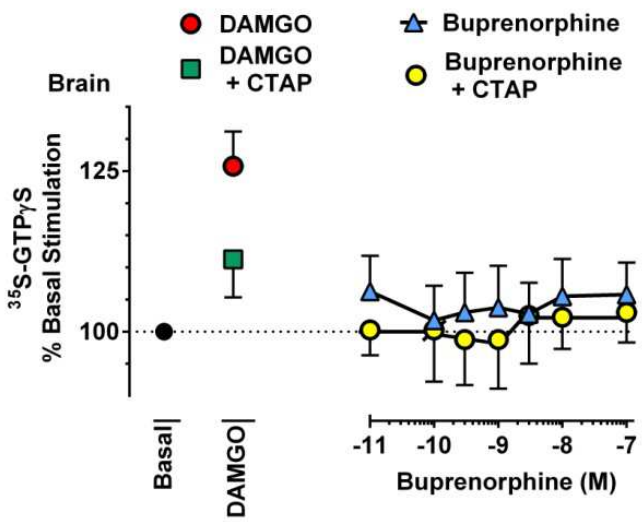


Figure 31: Effect of buprenorphine on ^{35}S-GTPγS binding in C57 mouse brain.
^{35}S-GTPγS binding assays were performed with membrane homogenate from C57 mouse brain. Although DAMGO stimulated ^{35}S-GTPγS binding, buprenorphine did not appear active concentrations up to 100nM, a concentration which produced near-maximal effects in cell lines. The addition of CTAP failed to unmask any inverse agonist effect produced by kappa opioid receptors, although it substantially lowered DAMGO stimulation. Results are pooled from 5 independent replicates, each showing similar results, and are expressed as mean ± SEM.

stimulation of ^{35}S-GTPγS binding in mouse brain homogenate despite its robust stimulation in mMOR expressing cell lines and the ability of DAMGO to stimulate ^{35}S-GTPγS binding in the same tissue. These results could not be explained by a summation of agonist and inverse agonist effects at different receptors as addition of CTAP failed to unmask inverse agonist effects as would be predicted by blocking mu receptors alone. This data is consistent with previous reports (Romero et al., 1999), and points to the limits of simplistic *in vitro* models of complex opioid receptor pharmacology *in vivo*, especially for a drug which is known to require multiple splice variants of mu receptors to produce maximal effects. Unfortunately, the failure of buprenorphine to stimulate ^{35}S-GTPγS binding even in wildtype brain homogenate precluded any comparison of differences in MOR exon 11 knockout brain.

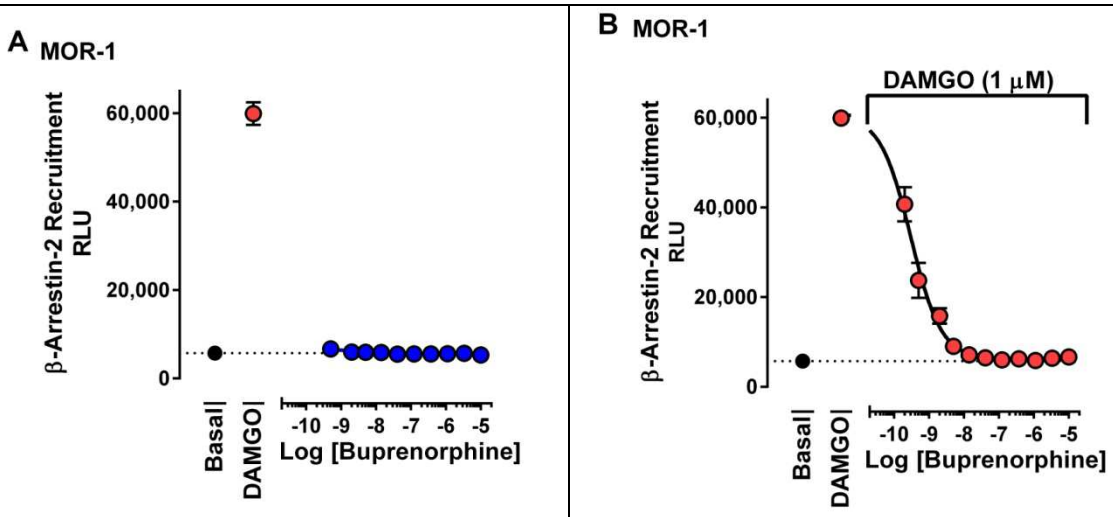


Figure 32: Effect of buprenorphine on recruitment of β-arrestin-2.
(A) Buprenorphine failed to stimulate recruitment of β-arrestin-2 up to 10 μM concentrations in a PathHunter assay, despite robust recruitment by 1uM DAMGO. (B) Buprenorphine potently antagonized the β-arrestin-2 recruitment produced by a fixed dose of 1μM DAMGO with an IC_{50} of 1.1 nM. Results are pooled from at least 2 independent experiments with similar results, and are expressed as mean ± SEM.

Finally, we sought to elaborate on previous investigations of buprenorphine's G-protein independent signaling via recruitment of β-arrestin-2 (McPherson et al., 2010). Biased agonists which preferentially activate G-protein signaling pathways have been suggested as superior pain relievers lacking side effects associated with arrestin signaling. Indeed, Phase II clinical trials are in progress with a G-protein biased mu opioid receptor agonist (TRV-130) for treatment of severe pain with a superior side effect profile such as decreased depression of respiration. If this hypothesis is correct, buprenorphine is the perfect biased agonist, with high potency in the ^{35}S-GTPγS assay and no detectable stimulation of β-arrestin-2 recruitment at concentrations of up to 10μM. In fact, buprenorphine potently antagonized β-arrestin-2 recruitment by DAMGO with an IC_{50} of just 1.1 nM.

Discussion

The development of a powerful pain reliever with an improved side effect profile over currently used opioid analgesics is the ultimate goal of opioid pharmacology research and decades of medicinal chemistry efforts. Indeed, with buprenorphine, that goal appears to have been partially realized, yet buprenorphine is still not commonly used by clinicians in the United States for the treatment of moderate to severe pain. This situation appears to be changing as buprenorphine's unique properties achieve more widespread appreciation and misconceptions are dispelled about its mu partial agonist classification in *in vitro* assays versus full agonist activity in humans with pain relief as an endpoint. Critically, recent studies have supported an additional role for buprenorphine's inverse agonism at kappa receptors in the treatment of anxiety and depression and in combating relapse in opioid and cocaine addicts (Cordery et al., 2014; Sorge, Rajabi, & Stewart, 2005; Wee, Vendruscolo, Misra, Schlosburg, & Koob, 2012).

This study examined the contribution of mu opioid receptor splice variants to the analgesic as well as the remaining side effects of buprenorphine. Consistent with previous findings, buprenorphine's analgesia is entirely mu receptor dependent, as exon 1/exon 11 double knockout animals show no analgesic response while DOR and KOR knockout animals show no difference from wildtype controls. Additionally, buprenorphine's analgesia is entirely MOR exon 11-dependent, as doses >300-fold higher than the analgesic ED_{50} in wildtype animals did not produce an analgesic response in the tail-flick test. Interestingly, exon 1-associated variants of the mu receptor are required for full analgesic response, as both MOR exon 1 and Triple opioid receptor knockout animals lacking exon 1-associated variants but retaining exon 11-associated variants produced a partial response plateauing at ~40% MPE.

Despite failing to produce an appreciable analgesic response in exon 11-knockout animals, buprenorphine's ability to antagonize morphine analgesia is consistent with its competition for the traditional mu opioid receptor sites mediating morphine analgesia. This model is conceptually similar to the as-yet unexplained finding that IBNtxA appears to bind traditional mu opioid receptors and acts as an agonist in ^{35}S-GTPγS assays in cell lines stably expressing mMOR-1 receptors yet fails to produce analgesia in exon 11-knockout animals. An alternate explanation could involve buprenorphine activating anti-analgesic or pro-nociceptive pathways, an effect mediated by ORL_1 receptors and believed to be responsible for the bell-shaped dose response curve observed with high doses of buprenorphine (Lutfy et al., 2003).

Indeed, the ^{35}S-GTPγS studies here are a cautionary tale for over-interpretation of the predictive value of *in vitro* data. In our hands, buprenorphine appeared to act as a full agonist in cell lines stably expressing mMOR-1 receptors, while others have reported observing lower efficacy (Romero et al., 1999; Selley et al., 1997). These differences may be easily explained by the differences in receptor number and G-protein expression levels between labs, especially as clonal populations are generally selected on the basis of opioid radioligand binding levels with no regard for levels of other proteins involved in signaling. Even within a clonal cell population, passage number and differences in cell culture technique and conditions can lead to significant differences in these parameters within a single lab. However, previous reports are in agreement with our findings that stimulation of ^{35}S-GTPγS binding is virtually absent in brain tissue despite its easily observed stimulation in cell lines. Although this has been hypothesized to reflect considerably lower receptor density in brain tissue relative to heterologously expressing cell lines, buprenorphine failed to appreciably stimulate^{35}S-GTPγS binding even in mu opioid receptor dense regions such as striasomal patches in the striatum and

antagonized morphine stimulation of ^{35}S-GTPγS binding as potently as the full antagonist CTAP (Romero et al., 1999). We submit an alternate explanation on the basis of our *in vivo* data - that these results are perfectly consistent with a model in which buprenorphine analgesia is mediated via a seperate, MOR exon 11-associated target, but acts as a weak partial agonist at the MOR exon 1-associated site mediating morphine's analgesia.

On the surface, there are many similarities between IBNtxA and buprenorphine - indeed buprenorphine exhibited very high affinity for the site labeled by [^{125}I]IBNtxA in both triple knockout mouse and rat brain. It is therefore tempting to speculate that the partial analgesic response observed in MOR exon 1 and Triple Knockout Mice is mediated by the same receptor as IBNtxA. However, buprenorphine clearly differs from IBNtxA in that full analgesic response does require exon 1-associated variants. However, the loss of analgesia observed in MOR exon 11-knockout animals clearly differentiates this MOR exon 1-dependent target from that mediating the effects of morphine and methadone, which retain full analgesic efficacy in MOR exon 11-knockout animals. On the basis of these studies, we hypothesize the existence of at least 3 separate mu opioid targets: Exon 1-dependent, Exon 11-dependent, and Exon 1- and 11- dependent.

Critically, buprenorphine, although displaying a superior safety profile with regard to its respiratory depressant effects, still produces constipation – a frequently dose-limiting side effect of clinically used mu analgesics (Reimer et al., 2008). Despite loss of analgesia, MOR Exon 11-knockout animals continue to show inhibition of GI transit following buprenorphine administration, suggesting that this side effect is not mediated by the same target as that producing analgesia. The locomotor stimulating effects were also completely intact, suggesting that buprenorphine's disinhibition of mesolimbic dopamine release was also mediated independently of its analgesia. As the

common pathway by which drugs of abuse produce reward, these findings support further investigation using conditioned place preference or self-administration in order to determine whether exon 11-associated variants also dissociate buprenorphine analgesia from reward.

Chapter 5: Kappa$_1$, and Alpha$_2$-adrenergic receptor agonists require truncated 6-transmembrane variants of the mu opioid receptor for analgesia but not side effects

Introduction

Achieving the goal of powerful pain relief without side effects has led many researchers to abandon mu opioids altogether, rather than pursuing a strategy of subtype selectivity. Early promise was seen with what we now know are full agonists at kappa$_1$ opioid receptors such as nalorphine – however, tests in humans showed they had their own significant side effects such as psychotomimesis and dysphoria (Cahal, 1957; Kumor et al., 1986). While partial kappa agonists show a reduced incidence of these side effects, their use is a dwarfed by that of traditional mu agonists like morphine, fentanyl, oxycodone, and hydrocodone.

Alpha2 adrenergic agonists such as xylazine are widely used in veterinary analgesia, while clonidine and especially dexmedetomidine are increasingly used for their sedative and analgesic properties in surgical and intensive care settings. Systematic review and meta-analysis of randomized, placebo controlled trials of clonidine or dexmedetomine administered to surgical patients found that alpha2 agonist treatment decreased post-operative pain intensity, lowered opioid consumption, and decreased incidence of opioid-related adverse events such as nausea (Blaudszun, Lysakowski, Elia, & Tramèr, 2012). Paul and Tran showed analgesic crosstolerance between clonidine and nalorphine, a kappa$_3$ opioid agonist which we previously showed to require exon 11-associated MOR splice variants for full analgesic potency (Majumdar, Grinnell, et al., 2011; Paul & Tran, 1995). Analgesic synergy between the alpha2 adrenergic system and the opioid system has been studied in depth (for a recent review, see Chabot-Doré, Schuster, Stone, & Wilcox, 2014). Synergy is believed to occur on multiple levels, with

interactions occurring by direct physical interactions between receptors (Vilardaga et al., 2008) as well as N- and P-type calcium channels and Protein Kinase C, but not Protein Kinase A (Roerig & Howse, 1996; Wei, Karim, & Roerig, 1996; Wei & Roerig, 1998).

As with the alpha2-adrenergic receptors, opioid-cannabinoid system crosstalk has been well established (for a recent review, see Katia, 2015). These interactions are also believed to occur at multiple levels, including via intracellular messengers, downstream release of endogenous ligands, and possibly even direct protein-protein interactions. Cannabinoids such as Δ9-tetrahydrocannabinol and nabilone, as well as *Cannabis sativa* itself, are used to treat pain, either alone or in combination with opioid pain relievers, although their abuse potential and significant effects on cognition also limit their use. Still, increasingly relaxed state laws and federal enforcement have led to a dramatic increase in patients using cannabis for the treatment of pain and countless other indications in the last decade – a trend that seems unlikely to reverse in the forseeable future.

Following our unexpected findings that buprenorphine analgesia was totally lost in MOR exon 11 knockout animals, we questioned whether other established classes of analgesics, opioid and non-opioid alike, might also unexpectedly require MOR exon 11-associated splice variants.

Results

Effect of MOR exon 11 knockout on kappa$_1$ opioid agonist behavioral pharmacology

In wildtype C57Bl/6 animals, the prototypical kappa$_1$-selective agonist U50,488 was a potent analgesic when administered systemically, supraspinally, or spinally (Figure 33A-C). However, we observed a dramatic loss in analgesic potency in MOR exon 11 knockouts, with animals failing to demonstrate even 25% response at doses greater

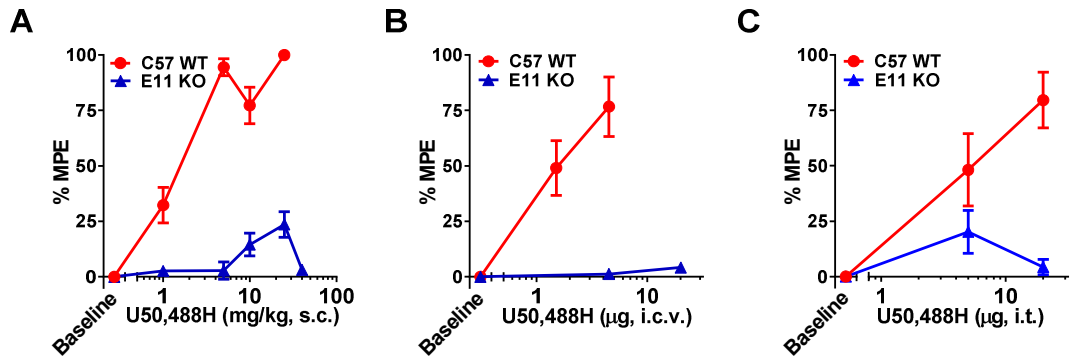


Figure 33: Effect of MOR exon 11 knockout on U50,488H analgesia.
Wildtype C57BL/6 (C57) or MOR exon 11 knockout (E11 KO) mice were injected via the indicated route with escalating doses of U50,488H and tested using a radiant heat tail flick assay at peak effect. Results are mean ± SEM from at least 2 independent experiments, each producing similar results. (A) Subcutaneous injection (n = 10-19 for each group): ED_{50} values (95% confidence limits) were 1.7 mg/kg (1.1 – 2.6) for C57. No reliable ED_{50} could be obtained for the E11 KO group, but there was a significant difference between genotypes ($p < 0.0001$ for a main effect of genotype, 2-way ANOVA; $p < 0.05$ at 1 mg/kg dose and $p < 0.0001$ for 5, 10, and 20 mg/kg doses, post-hoc Bonferroni multiple comparison test). (B) Intracerebroventricular injection (i.c.v., n = 9 for C57, n = 7 for E11 KO): The ED_{50} for C57 animals (95% confidence limits) was 1.5 ug (0.76 – 2.9). However, E11 KO animals failed to display any significant response doses >13-fold higher than the wildtype ED_{50}. At the highest dose tested in wildtype animals, there was significantly lower analgesic response in E11 KO animals vs C57 ($p = 0.002$, t-test). (C) Intrathecal injection (i.t., n = 5 for each group): The ED_{50} for C57 animals (95% confidence limits) was 5.3 ug (2.4 – 12). It was not possible to determine a reliable ED_{50} in E11 KO animals, but there was significantly lower analgesic response in E11 KO animals vs C57 ($p = 0.0001$ for a main effect of genotype, 2-way ANOVA; $p < 0.003$ at 5ug dose and $p < 0.0001$ at 20 ug dose, post-hoc Bonferroni multiple comparisons test).

than 30-fold higher than the ED_{50} obtained in wildtype controls when injected subcutaneously. Supraspinally, U50,488 analgesia was completely abolished in MOR exon 11 knockout animals at doses up to 13-fold greater than the wildtype ED_{50}. Spinally, knockout animals failed to reach 25% maximum possible effect at doses 4-fold higher than wildtype ED_{50}.

The natural product and neoclerodane diterpene Salvinorin A has also been shown to be highly selective for the KOR (Roth et al., 2002), despite its obvious structural dissimilarity to either the prototypic selective arylacetamide kappa opioid agonists such as U50,488 and U69,593 or the non-selective benzomorphans such as ketocyclazocine which originally led Martin to propose the existence of a distinct population of "kappa" opioid receptors (Martin et al., 1976). Consistent with the results observed for U50,488, we observed a significant loss of Salvinorin A analgesia in MOR exon 11 knockout animals relative to wildtype controls (Figure 34), suggesting that the results are applicable to the kappa opioid system and are not peculiar to the arylacetamide class of kappa agonists.

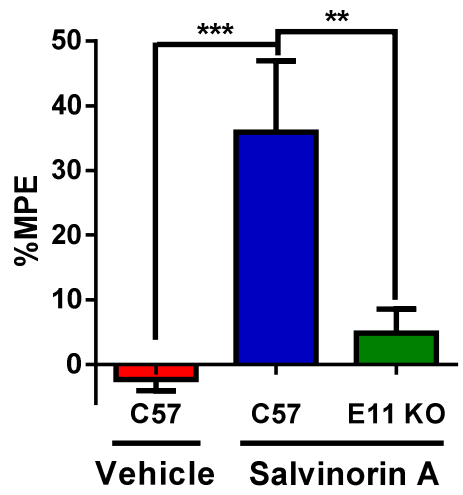


Figure 34: Effect of MOR exon 11 knockout on Salvinorin A analgesia.
Wildtype C57BL/6 (C57, n = 10) or MOR exon 11 knockout (E11 KO, n = 19, 2 independent replicates) mice were injected with a 10 mg/kg dose of Salvinorin A and tested using a radiant heat tail flick assay 10 minutes later at peak effect. Salvinorin A produced modest but significant increase in tail flick latency in C57 animals ($p < 0.001$ vs vehicle control in the same animals) but was without significant effect in E11 KO animals ($p = 0.67$ vs vehicle control, One-way ANOVA with post-hoc Tukey multiple comparison test). There was a significant difference between C57 and E11 KO analgesic response ($p < 0.002$).

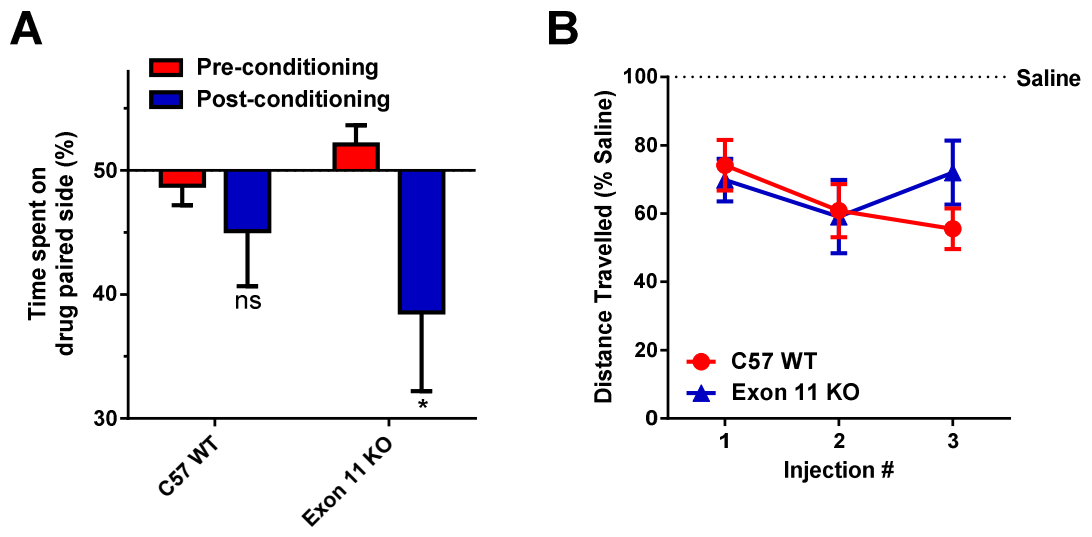


Figure 35: Effect of MOR exon 11 knockout on U50,488H conditioned place aversion and locomotor behavior.
(A) E11KO animals (n = 14 for each group) showed a significant aversive response to a moderate 5 mg/kg s.c. dose of U50,488 in a conditioned place aversion test (Post-conditioning vs. pre-conditioning, $p < 0.027$, Bonferroni multiple comparisons post-hoc test after 2-way repeated measures ANOVA). In our apparatus, the response to this conditioning/dose in wildtype C57 animals failed to reach significance ($p = 0.21$). (B) There was no difference in U50,488's depression of wildtype C57 and E11 KO animals' locomotor behavior during any of the 3 drug conditioning sessions relative to morning saline control ($p = 0.62$ for genotype, $p = 0.34$ for session number, $p = 0.40$ for interaction, 2-way ANOVA).

The psychotomimetic and aversive effects of kappa opioid agonists have substantially limited their development and use as analgesics, and the endogenous kappa opioid receptor system has been shown to mediate the aversive effects of stress and stress-induced reinstatement of drug seeking, a model of relapse in human addicts (Bruchas, Land, & Chavkin, 2010; Land et al., 2009; McLaughlin, Land, Li, Pintar, & Chavkin, 2006). We therefore sought to determine whether the aversive effects of a selective kappa agonist would also be impaired in MOR exon 11 knockout animals. To the contrary, we observed significant conditioned place aversion to U50,488 in knockout

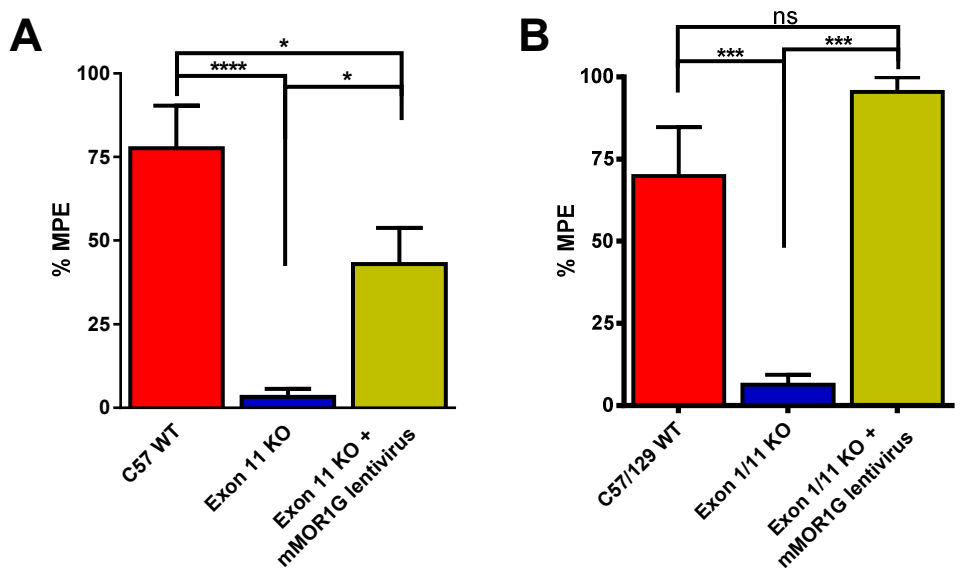


Figure 36: Lentiviral mMOR-1G rescue of U50,488H analgesia.
A lentivirus expressing EGFP and mMOR-1G was injected i.c.v. into MOR Exon 11 KO (A) or MOR Exon 1/11 KO (B). U50,488H (20 mg/kg, s.c.) analgesia tested at least 5 weeks later. Results are mean ± SEM from at least 2 independent experiments, each showing similar results. (A) Consistent with previous findings, Exon 11 KO (n = 10) showed significantly impaired analgesic response to a 20 mg/kg dose of U50,488H ($p < 0.0001$ vs C57 WT (n = 9), post-hoc Tukey test following one way ANOVA). Significant analgesic efficacy was restored following lentiviral mMOR-1G infection (n = 8, $p < 0.05$ vs E11KO) although analgesia was still reduced relative to wildtype control ($p < 0.05$). (B) As predicted, Exon 1/11 KO (n = 10) showed significantly impaired analgesic response to a 20 mg/kg dose of U50,488H ($p < 0.0005$ vs C57/129 WT (n = 8), post-hoc Tukey test following one way ANOVA). Analgesic efficacy was fully restored following lentiviral mMOR-1G infection (n = 3, $p < 0.001$ vs E1/E11KO; $p = 0.37$ vs C57/129 WT).

animals ($p < 0.027$) even at a moderate dose of 5 mg/kg s.c. which produced no analgesia in knockout animals (Figure 35). Indeed, the difference observed in wildtype controls' post-conditioning preference did not reach significance at this dose in our apparatus, confirming that the knockout animals retain equal or greater sensitivity to the aversive properties of kappa agonists. We also observed that U50,488 depressed locomotor behavior to a similar extent in both genotypes across each of 3 drug-training

sessions relative to saline (Figure 36). Together, these results implicate exon 11-associated variants in the analgesia, but not locomotor depressing or aversive side effects of kappa$_1$ opioid agonists.

Finally, rescue experiments were performed using a mMOR-1G lentivirus (Lu et al., 2015), in press). As previously observed, both MOR Exon 11 knockout and MOR Exon 1/11 Knockout animals showed total loss of analgesia to 20 mg/kg U50,488H, but knockout animals infected with mMOR-1G lentivirus showed restored analgesic efficacy, suggesting that the truncated 6TM variant mMOR-1G is sufficient to restore kappa$_1$ opioid analgesia in animals with exon 11 knockout animals with intact KOR receptors.

Effect of MOR exon 11 knockout on alpha2 adrenergic agonist behavioral pharmacology

We next examined the role of exon 11-associated MOR splice variants in alpha2 agonist analgesia (Figure 37). In fact, MOR exon 11 knockout mice showed a significant impairment in tail flick analgesia relative to their wildtype controls following clonidine administration by multiple routes, although analgesia was not completely lost. Systemically, a very steep dose-response curve was observed, with clonidine analgesia failed to exceed 20% MPE at >2 times the ED_{50} in wildtype animals. The difference between genotypes was even more evident supraspinally, where a dose >30-fold higher than the ED_{50} in the wildtype C57 animal produced only 23% MPE response. However, spinally, there seemed to be some intact analgesic mechanism remaining, although approximately 12-fold rightward shift in ED_{50} was observed.

We next tested dexmedetomidine, another imidazoline alpha$_2$ adrenergic receptor agonist which is more selective for the alpha$_{2a}$ subtype than clonidine (Figure 38). In wildtype animals it was a very potent analgesic in the tail flick assay, with an ED_{50}

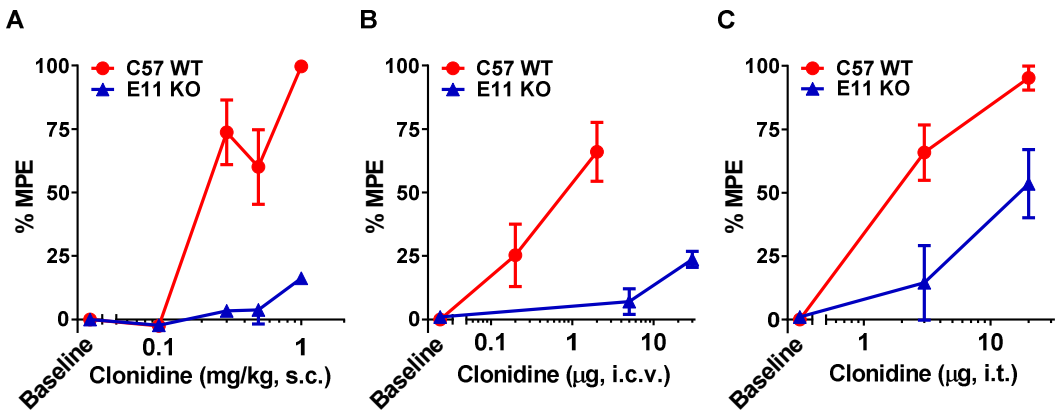


Figure 37: Effect of MOR exon 11 knockout on clonidine analgesia.
Wildtype C57BL/6 (C57) or MOR exon 11 knockout (E11 KO) mice were injected via the indicated route with escalating doses of the prototypical Alpha2 adrenergic receptor agonist clonidine and tested using a radiant heat tail flick assay 30 minutes later at peak effect. Results are mean ± SEM and are the results from at least 2 independent experiments, each producing similar results. (A) Subcutaneous injection (n = 5-10 for C57, n = 10 for E11KO): ED50 value (95% confidence limits) for C57 animals was 0.30 mg/kg (0.19 – 0.46). It was not possible to determine a reliable ED50 in E11 KO animals at the doses tested, but there was significantly lower analgesic response in E11 KO animals vs C57 ($p = 0.0001$ for a main effect of genotype, 2-way ANOVA; $p < 0.0001$ at 0.3, 0.5, and 1 mg/kg doses, Bonferroni multiple comparisons post-hoc test). (B) Clonidine, intracerebroventricular injection (n = 10 for each group). The ED50 for C57 animals (95% confidence limits) was 0.84 ug (0.39 – 1.8). It was not possible to determine a reliable ED50 in E11 KO animals at the doses tested, but dose response curves were significantly different ($p < 0.0001$, extra sum-of-squares F test). (C) Clonidine, intrathecal injection (n = 5 for each group). ED50 values (95% confidence limits) were 1.5 ug (0.84 - 2.7) for C57 vs 18 mg/kg (8 - 42) for E11 KO, which were significantly different ($p < 0.0001$, extra sum-of-squares F test).

(95% CI) of 0.4 mg/kg (0.02 – 0.06). As predicted, analgesia was substantially impaired in MOR Exon 11 knockout animals, consistent with a loss of $alpha_{2a}$-mediated analgesia.

The sedative properties of alpha2 agonist drugs such as dexmedetomidine are often desirable in the surgical and intensive care settings where they are currently employed; however, wider use of this class of drugs for the treatment of opioid-refractory pain is limited by this same effect outside of a hospital context (Jackson,

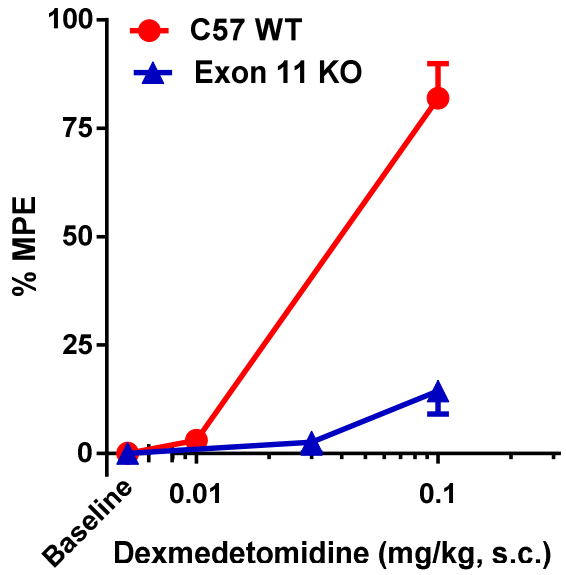


Figure 38: Effect of MOR exon 11 knockout on dexmedetomidine analgesia.
Wildtype C57BL/6 (C57, n = 9-13) or MOR exon 11 knockout (E11 KO, n = 6-10) mice were injected with escalating doses of dexmedetomidine and tested using a radiant heat tail flick assay 30 minutes later at peak effect. Results are mean ± SEM and are the results at least 2 independent experiments, each producing similar results. ED_{50} value (95% confidence limits) for C57 animals was 0.04 mg/kg (0.02 - 0.06). E11 KO response at the 0.1 mg/kg dose was significantly lower than C57 WT ($p < 0.0001$, 2-tailed t-test).

Wohlt, and Fine 2006). We therefore sought to determine whether the locomotor depressant actions of alpha2 agonists were impaired as was analgesia in MOR exon 11 knockout animals (Figure 39). As with delta and kappa agonists, despite profound loss of analgesic potency, a moderate dose of clonidine (0.3 mg/kg s.c.) still significantly depressed total distance travelled during a 1 hour session in an open field activity chamber in knockout animals as in wildtype C57 controls. Furthermore, there was no difference in distance travelled between genotypes following either saline or clonidine, and no significant difference was seen between the genotypes' reaction to either saline or clonidine at any time point during the 60 minute session.

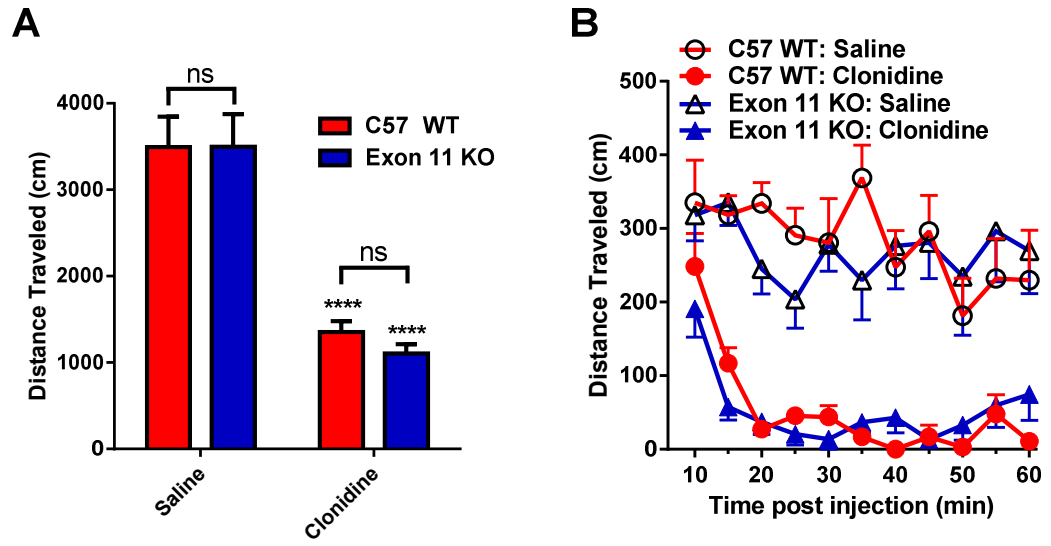


Figure 39: Effect of MOR exon 11 knockout on the locomotor depressing effects of clonidine.

Animals were injected with a moderate dose clonidine (0.3 mg/kg, s.c.), and their locomotor behavior was recorded for the following 60 min in an open field locomotor chamber. Clonidine significantly reduced depressed locomotor behavior relative to saline, but there was no difference between each genotype's response ($p < 0.0001$ for a main effect of drug, $p = 0.65$ for a main effect of genotype, $p = 0.65$ for an interaction, 2-way repeated measures ANOVA; $p < 0.0001$ for each genotype's response to clonidine vs. saline, but $p > 0.99$ for a difference between genotypes for saline and $p > 0.98$ for a difference between genotypes for clonidine, post-hoc Bonferroni multiple comparisons test).

Finally, rescue experiments were performed using the mMOR-1G lentivirus to restore expression of a truncated 6TM MOR isoform. As previously observed, both MOR Exon 11 knockout and MOR Exon 1/11 Knockout animals showed total loss of analgesia to 0.5mg/kg clonidine, and MOR Exon 11 knockout animals infected with mMOR-1G lentivirus showed restored analgesic efficacy. Additionally, MOR exon 1/11 animals showed an analgesic response that was not significantly different from wildtype, although this response narrowly failed to reach significance ($p < 0.054$) likely due to the

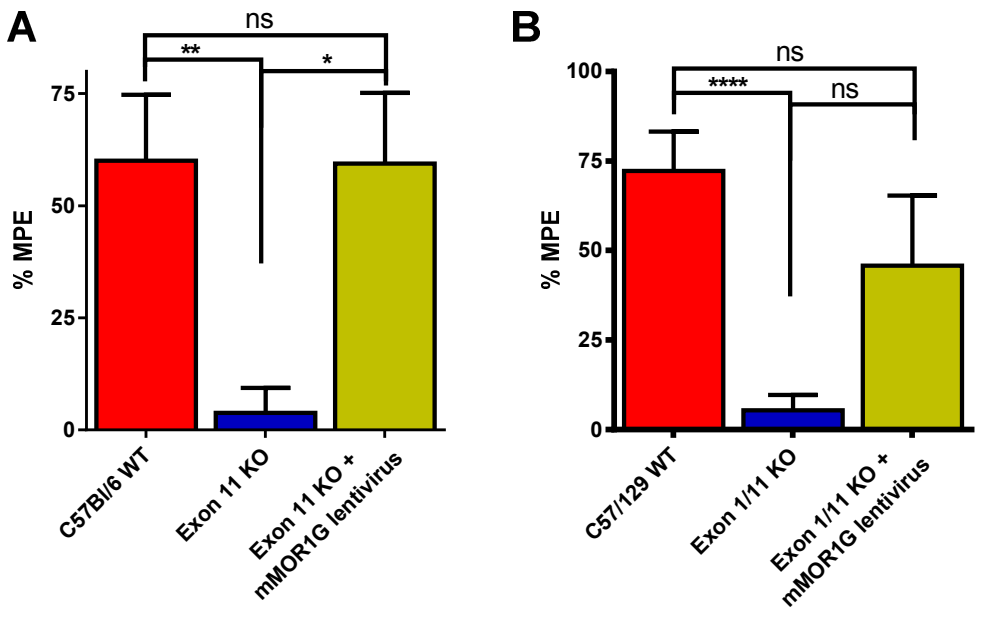


Figure 40: Lentiviral mMOR-1G rescue of clonidine analgesia.
A lentivirus expressing EGFP and mMOR-1G was injected i.c.v. into MOR Exon 11 KO (A) or MOR Exon 1/11 KO (B). U50,488H (20 mg/kg, s.c.) analgesia tested at least 5 weeks later. Results are mean ± SEM from at least 2 independent experiments, each showing similar results. (A) Consistent with previous findings, Exon 11 KO (n = 10) showed significantly impaired analgesic response to a 0.5 mg/kg dose of clonidine ($p < 0.01$ vs C57 WT (n = 10), post-hoc Tukey test following one way ANOVA). Clonidine analgesia was fully restored following lentiviral mMOR-1G infection (n = 8, $p < 0.05$ vs E11KO; $p > 0.99$ vs C57 WT) (B) As predicted**,** Exon 1/11 KO (n = 10) showed significantly impaired analgesic response to a 20 mg/kg dose of U50,488H ($p < 0.0005$ vs C57/129 WT (n = 8), post-hoc Tukey test following one way ANOVA). Following lentiviral mMOR-1G infection, clonidine analgesia did not differ significantly from C57/129 WT (n = 3, $p = 0.27$); however, it narrowly failed to reach significance relative to MOR E1/E11 KO ($p < 0.054$ vs E1/E11KO).

small number of knockouts (n = 3) available to test. Further experiments will be pursued as more animals become available in the future. In total, these results confirm that truncated 6TM variants such as mMOR-1G, are required for the expression of alpha$_2$ adrenergic agonist analgesia.

Lack of effect of MOR exon 11 knockout on cannabinoid type 1 agonist analgesia

Finally, we examined the role of exon 11-associated MOR splice variants in Cannabinoid type 1 agonist analgesia. Unlike delta (not shown) and kappa opioid agonists or alpha$_2$ adrenergic agonists, however, we did not observe any significant difference in analgesic response between MOR exon 11 knockout animals and wildtype controls to the selective CB_1 agonist CP55,940. In fact, the response was virtually indistinguishable between groups, offering a robust negative control and confirmation that MOR exon 11 knockout does not produce any form of global deficit in analgesic response.

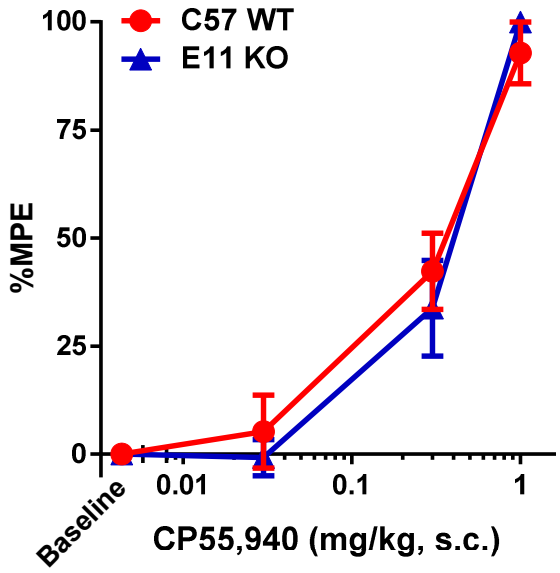


Figure 41: Lack of effect of MOR exon 11 knockout on cannabinoid type 1 agonist analgesia.
Wildtype C57BL/6 (C57) or MOR exon 11 knockout (E11 KO) mice (n = 6-13 for each group) were injected s.c. with escalating doses of the prototypical Cannabinoid type 1 receptor agonist, CP55,940, and tested using a radiant heat tail flick assay 30 minutes later at peak effect. Results are mean results of 2 independent experiments, each producing similar results. ED_{50} values (95% CI) were 0.32 (0.20 – 0.52) for C57 WT and 0.37 (0.21 – 0.66) for E11 KO. There was no significant difference between EC_{50} values between genotype (p = 0.64)

Discussion

Following the unexpected loss of buprenorphine analgesia in MOR exon 11 knockouts, we examined kappa$_1$, alpha$_2$ adrenergic, and cannabinoid type 1 agonist analgesia to determine whether they would also be affected. Indeed, the kappa agonist U50,488H showed a complete loss of analgesic efficacy at doses many times higher than wildtype ED_{50} by systemic, supraspinal, or spinal administration. Systemic administration of the structurally-unique, non-nitrogenous, and recreationally-used kappa agonist Salvinorin A mimicked our findings with U50,488H. Lentiviral delivery of mMOR-1G – a 6TM splice variant of the mu opioid receptor found in the mouse, rat, and human genome – restored analgesic efficacy of U50,488H in two different knockout models, confirming the involvement of these isoforms. Critically, the aversive and locomotor depressing actions of U50,488H were fully intact in MOR Exon 11 knockout animals, suggesting that these effects can be genetically dissociated and that it may be possible to design psychologically benign kappa$_1$ analgesics or selectively antagonize kappa-mediated dysphoria without perturbing pain-related circuits.

Similar results were found for the alpha$_2$ adrenergic agonist clonidine, although the loss of analgesia appeared to be more pronounced systemically and supraspinally than spinally where only a 12-fold shift in analgesia was observed. These results would be consistent with loss of alpha$_{2A}$-mediated analgesia, while at higher doses clonidine can interact with alpha$_{2C}$ subtypes present in the spinal cord which are also capable of producing analgesia (Fairbanks et al., 2002). Dexmedetomidine analgesia was also lost, consistent with the involvement of alpha$_{2A}$ subtypes for which it displays higher selectivity. This model could be further tested using alpha$_2$-subtype knockouts as the available agonists possess only moderate selectivity for one receptor over another. As

with kappa$_1$ receptors, analgesic efficacy was rescued by lentiviral delivery of mMOR-1G, confirming the involvement of truncated 6TM receptor isoforms.

Finally, as with U50,488, the locomotor depressing side effects of clonidine were fully intact despite loss of analgesic efficacy, dissociating these effects from the analgesic effects of this class of drugs. Clonidine has a dramatically different mechanism of depressing locomotor activity than U50,488, depressing noradrenergic output from the locus ceruleus globally by activating autoreceptors on efferent neurons (Gilsbach et al., 2009). Critically, the analgesic effects of clonidine and dexmedetomidine were found to be mediated by postsynaptic receptors on non-adrenergic neurons, such as primary nociceptors, in spinal pain relay neurons, and excitatory interneurons in the dorsal horn (Pertovaara, 2006). These correlations between location and function may be of assistance in assigning the location of the interaction between systems.

Loss of kappa$_1$ and alpha$_2$ agonist agonist analgesic response is difficult to explain for several reasons. No difference in kappa opioid receptor affinity or abundance was observed in MOR exon 11 knockout mouse brain with the kappa agonist [^{3}H]-U69,593 (Pan et al 2009). Kitchen et al found no alteration in kappa affinity or expression pattern in the Kieffer lab MOR knockout made by disrupting exons 2, which is shared by both 6TM and 7TM isoforms (Kitchen, Slowe, Matthes, & Kieffer, 1997). These studies are not necessarily in conflict with our findings, where we can conceive of a model whereby the 6TM receptor permits the kappa receptor to signal more efficiently or via a different effector pathway, resulting in analgesia. This would further explain why analgesia is lost but other effects remain unaffected in the absence of these truncated isoforms. The impact of the different MOR-1 knockout models on MOR-1 splicing has not been systematically studied, so it is impossible to know which splice variants would be retained in each animal.

More difficult to reconcile are results showing the Kieffer lab exon 2 knockout expressed no loss of sensitivity to kappa agonists although delta agonist analgesia was diminished (Matthes et al., 1998). Additionally, an MOR exon 2/3 knockout from the Loh group showed no analgesic difference from wildtype following administration of the alpha2 agonist UK14,304 (Guo, Fairbanks, Stone, & Loh, 2003). The results from these dramatically different knockout models appear to be conflicting, however we believe the lentiviral rescue experiments in 2 separate knockout models offer compelling evidence for an involvement of 6TM receptors.

Unlike $kappa_1$ and $alpha_2$ agonist analgesia, cannabinoid type 1 agonist analgesia appeared totally unaffected by loss of MOR exon 11 splice variants. Indeed, this was a welcome development as it argued against a global analgesic deficit and offered a much-needed negative control in addition to morphine and methadone.

Following our unexpected findings with buprenorphine, it was perhaps less surprising but all the more exciting that other receptor systems showed a profound loss of analgesic response in MOR exon 11 knockout animals. It was even more exciting to discover that some of the major side effects which have precluded more widespread use of agonists targeting these systems were intact in these animals. Taken together, these results suggest that the targets mediating the desired effect – analgesia – are distinct and genetically dissociable from the targets mediating major undesired effects of the drugs.

Chapter 6: Synthesis of Novel Opioid Photoaffinity Labels

Introduction

Affinity labeling is a classical biochemical technique which facilitates the visualization and identification of the molecular target of a pharmacologically defined macromolecular binding site. Early attempts at mapping enzyme active sites successfully used small molecules such as phenylmethanesulfonylfluoride (PMSF) to selectively and covalently label the catalytic serine of the protease chymotrypsin (Balls & Jansen, 1952). However, this approach relied on the exceptional reactivity of this particular serine, while other macromolecules such as receptors or antibodies do not possess an analogous reactive residue. To this end, the general method of affinity labeling was conceived and its usefulness demonstrated using the reactive diazonium fluoroborate derivative of a *p*-nitrophenylarsenate hapten to covalently label the "active site" of a rabbit antibody to benzenearsonic acid (Wofsy, Metzger, & Singer, 1962).

Avram Goldstein's lab made pioneering attempts at developing a photoactivatable affinity label for opioid receptors in 1972 with *N*-*p*-(azidophenyl)-ethylnorlevorphanol; unfortunately, this ligand suffered from high levels of nonspecific binding which precluded its successful use. The labs of Portoghese and Takemori were more successful with their development a pair of agonist and antagonist alkylating agents, chloroxymorphamine and chlornaltrexamine, respectively, which were shown to irreversibly label opioid receptors and expressed extremely long-lasting actions *in vivo* consistent with this characterization (Caruso, Takemori, Larson, & Portoghese, 1979; Portoghese, Larson, Jiang, Caruso, & Takemori, 1979; Portoghese, Larson, Jiang, Takemori, & Caruso, 1978; Takemori, Larson, & Portoghese, 1981). Portoghese's group synthesized another alkylating mu ligand, β-funaltrexamine, which displayed irreversible

antagonist activity at mu receptors (Portoghese, Larson, Sayre, Fries, & Takemori, 1980; Ward, Portoghese, & Takemori, 1982). This drug would later be used to stabilize the antagonist conformation of the mu receptor for crystallization (Manglik et al., 2012).

[^{3}H]-chlornaltrexamine was used in an attempt to visualize the molecular weight of opioid receptor complexes from mouse brain, giving 4 peaks after gel-filtration chromatography with 2 running together at a molecular weight of 590 kDa (Caruso, Larson, Portoghese, & Takemori, 1980). In contrast, Eric Simon's group had estimated the molecular weight of opioid receptor complexes to be approximately 400 kDa following reversible labeling with [^{3}H]-etorphine and purification under native conditions (Simon, Hiller, & Edelman, 1975). As we now know the molecular weights of mature, glycosylated, monomeric mu, delta, and kappa opioid receptors are in the range of 55-75 kDa, it is unclear whether these estimates reflect true signaling complexes, receptor homo/heteromers, aggregates, or are simply artefactual. High levels of nonspecific incorporation further complicate interpretation, and the tritium radiolabel makes monitoring purification difficult, and precludes exposing film following SDS-PAGE.

Kenner Rice also synthesized several mu- and delta-selective alkylating agents derived from fentanyl including (+)-*cis*-3-methylfentanyl isothiocyanate or "super-FIT" (Rice et al., 1983). Using this delta-selective probe, the Klee group was able to isolate highly purified delta opioid receptors from NG108-15 cells in a purification scheme consisting of affinity labeling, solubilization, wheat germ agglutinin affinity chromatography followed by anti-fentanyl immunoaffinity chromatography, and finally separation by SDS gel electrophoresis (Simonds, Burke, Rice, Jacobson, & Klee, 1985). This scheme revealed a glycosylated band with a molecular weight of approximately 58 kDa, consistent with the molecular weight of the glycosylated DOR-1 gene product which would be discovered 7 years later. Interestingly, Simonds et al note that the

receptor had “a strong tendency to dimerize, even in the presence of denaturing detergents, and… exists primarily as an oligomer in nondenaturing detergents.”

Photoaffinity labeling has the added benefit that the reactive group is not exposed until exposure to ultraviolet light, allowing the experimenter to maximize receptor occupancy to equilibrium before photolysis and uncaging of an extremely reactive intermediate. This minimizes nonspecific labeling by virtue of its high likelihood of forming a covalent bond with amino acid residues in the immediate vicinity of the group after photoactivation, although in reality some portion of the labeling occurs after dissociation and reassociation of the ligand (Ruoho, Kiefer, Roeder, & Singer, 1973). Interestingly, no especially reactive group need necessarily be incorporated for UV photoactivation, although photolabeling efficiencies may be low as a result; Standifer et al were able to photolabel mu, kappa$_1$, or kappa$_3$ receptors using the radioligand [^{3}H]-naloxone benzoylhydrazone, which contains no traditional photoreactive group at all (Standifer, Murthy, Kinouchi, Steele, & Pasternak, 1991).

The carbene-generating benzophenone and diazirine and nitrene-generating aryl azide are the most common photoreactive groups used, each with its own advantages and disadvantages such as size, photolabeling efficiency, and reactivity in the absence of UV photoactivation. The photochemistry of phenylazide and its phenylnitrene photolysis product has been extensively studied (shown in **Figure 42**; for a thorough review, see Gritsan & Platz, 2010). Although the *ortho*-iodo substituted phenyl azide derivative has not been studied as extensively, the rate constant for the reaction of the *para*-iodo didehydroazepine rearrangement product with diethylamine (a model nucleophile) was 2 orders of magnitude lower than the unsubstituted parent azide, suggesting that the presence of iodine on the ring is likely to adversely affect photolabeling efficiency (Y. Li, Kirby, George, Poliakoff, & Schuster, 1988).

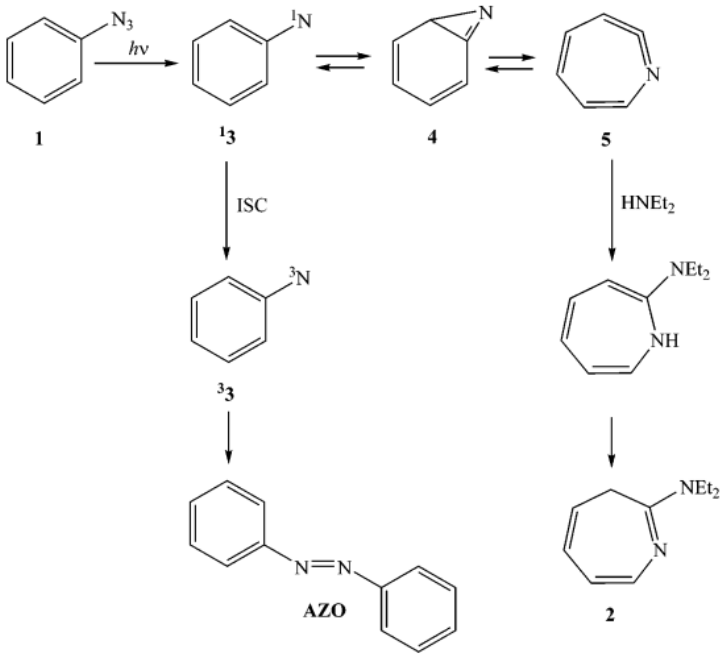


Figure 42: Photochemistry of phenyl azide
Following UV photolysis of phenylazide (1), molecular nitrogen is released to yield the highly reactive singlet phenylnitrene (^{1}3). Below 180K, spin relaxation via intersystem crossing (ISC) dominates to form the less reactive triplet nitrene (^{3}3), while at room temperature in solution rearrangement is preferred, forming the didehydroazepine (5) via a benzazirine (4) intermediate. In practice, it is this compound which forms covalent adducts (2) in the presence of nucleophiles, such as amines. Reproduced from (Gritsan & Platz, 2006).

Still, we chose the azide group due to its relatively small size and ease of synthetic incorporation into the IBNalA structure with the goal of producing a high affinity, high-specific activity photoaffinity label to facilitate purification and characterization of opioid receptors, including the target of IBNtxA.

Results

Two arylazide photoaffinity labels were synthesized by amide bond coupling between 4-azido-3-iodobenzoic acid and 6-β-naloxonamine (6) or *N*-propargyl-6-β-nornaloxonamine (7). Competition assays (Table 4 and Table 5) revealed that the both 6

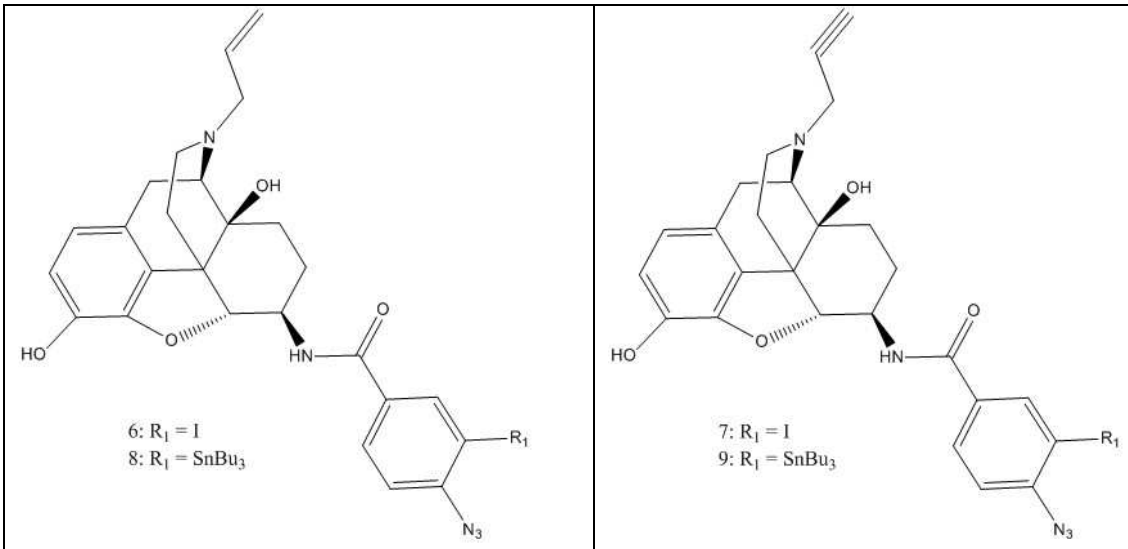


Figure 43: Structures of novel opioid photoaffinity labels.

Table 4: Competition studies of photoaffinity label 6

Escalating concentrations of **6** were incubated with in the presence of the indicated radioligand with membranes prepared from CHO cells stably transfected with each respective opioid receptor or triple knockout mouse brain.

Receptor	Membranes	Radioligand	K_i [95% CI] (nM)
Mu	CHO-MOR	^{3}H-DAMGO	0.20 nM [0.13 – 0.34]
Delta	CHO-DOR	^{3}H-DPDPE	16 nM [11 – 24]
Kappa	CHO-KOR	^{3}H-U69,593	0.26 nM [0.17 - 0.38]
6TM/E11	Mouse Brain (TKO)	^{125}I-IBNtxA	4.1 [3.5 – 6.4]

Table 5: Competition studies of photoaffinity label 7

Escalating concentrations of 7 were incubated with in the presence of the indicated radioligand with membranes prepared from CHO cells stably transfected with each respective opioid receptor or triple knockout mouse brain. Results are from 3 independent replications except for TKO mouse brain which is a single determination, and are presented as mean ± SEM.

Receptor	Membranes	Radioligand	K_i ± SEM (nM)
Mu	CHO-MOR	^{125}I-IBNtxA	0.25 ± 0.12
Delta	CHO-DOR	^{125}I-IBNtxA	3.5 ± 0.40
Kappa	CHO-KOR	^{125}I-IBNtxA	0.92 ± 0.29
6TM/E11	Mouse Brain (TKO)	^{125}I-IBNtxA	0.89 [0.71-1.1]

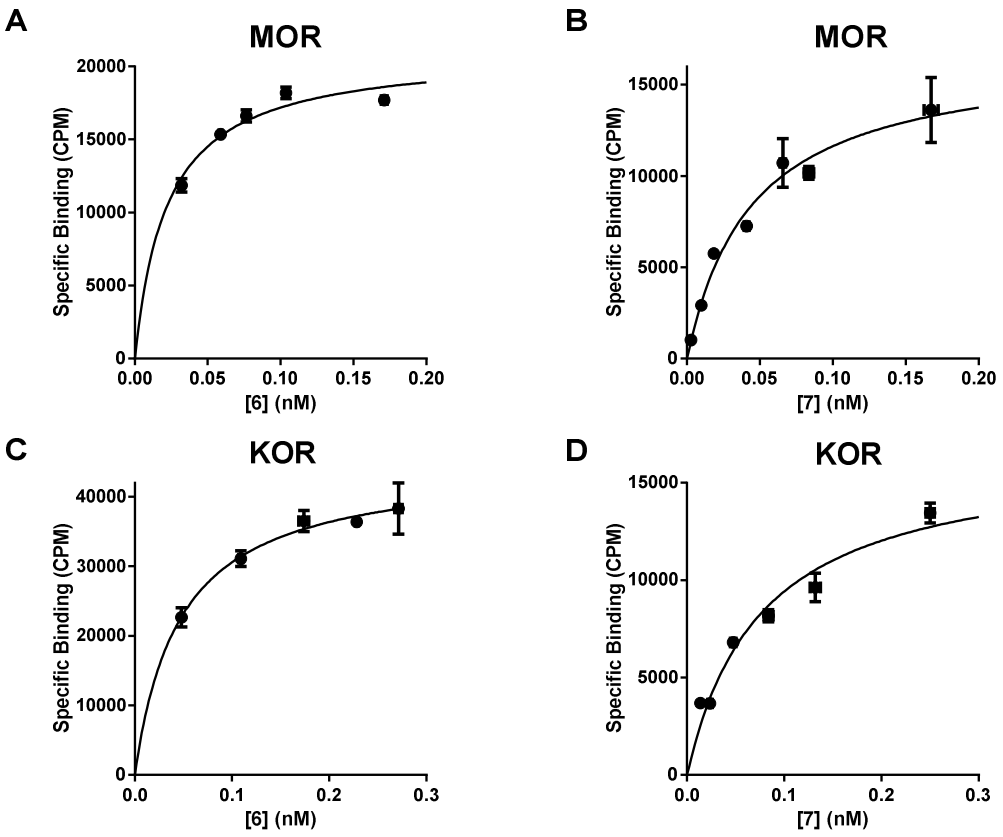


Figure 44: Saturation Binding Studies.
[^{125}I]-6 or [^{125}I]-7 incubated at the indicated concentrations with membrane homogenate from cells stably transfected with mMOR-1 or mKOR-1. Curves were fit by nonlinear regression using Graphpad Prism. Representative figures are shown. Each study was replicated 3 times except for and mean values for KD ± SEM of 3 independent replications were: CHO-MOR (A) 6: KD = 0.027 ± 0.004 nM and (B) 7: KD = 0.051 ± 0.013 nM. For CHO-KOR (C) 6: KD = 0.053 ± 0.002 nM (D) 7: KD = 0.056 ± 0.001 nM.

and 7 had moderate affinity for delta opioid receptors, and subnanomolar affinity for mu and kappa receptors suggesting the drugs could be useful for their desired purpose.

Following these initial promising findings, the corresponding radioiodination precursors were then prepared by coupling 4-azido-3-tributyltinbenzoic acid with 6-β-naloxonamine (8) or *N*-propargyl-6-β-nornaloxonamine (9). All structures and compound purity were confirmed by TLC, NMR, and LC/MS.

Reaction of the tributyltin precursors with chloramine T and $Na^{125}I$ and subsequent semi-preparative purification by reverse phase HPLC afforded [^{125}I]-6 and [^{125}I]-7. Saturation studies in CHO cells stably expressing mMOR-1 or mKOR-1 receptors revealed that the compounds even exhibited higher affinity for mu and kappa receptors than predicted based on competiton assays, with K_D values of [^{125}I]-6 for mu of 0.027 ± 0.004 nM and 0.053 ± 0.002 nM at kappa; K_D values of [^{125}I]-7 were about 2-fold lower for mu at 0.051 ± 0.013 nM although kappa affinity appeared relatively unchanged at KD = 0.056 ± 0.001 nM.

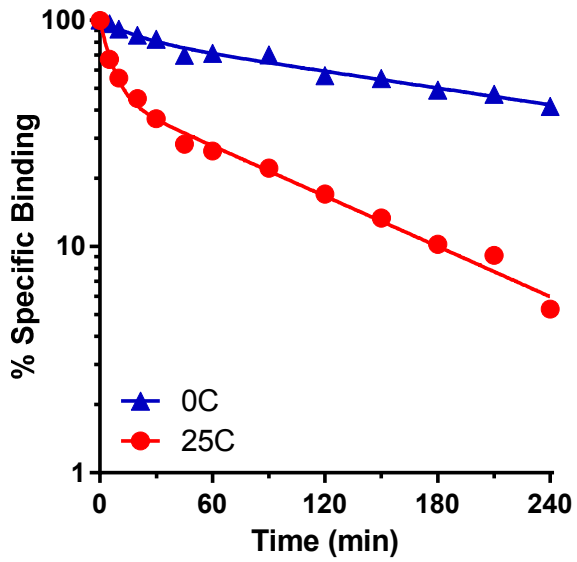


Figure 45: Dissociation studies of [^{125}I]-6
[^{125}I]-6 was incubated with CHO-MOR membranes at a concentration of 0.1 nM for 1.5 hours at 25C to reach equilibrium binding. 10uM levallorphan was then added at the indicated time points (5 – 240 min) to determine the kinetics of drug dissociation. Results shown are pooled from 3 independent replicates with similar results and are expressed as mean [95% confidence intervals]. At 25C, data was best fit by a 2-phase exponential decay model ($p<0.0001$ vs 1-phase decay model, Extra sum of squares F-test), with 53% [51 – 56] dissociating rapidly and the remainder very slowly. $t_{1/2\ Fast}$ = 4.6 min [4.1 – 5.2 min], $t_{1/2\ Slow}$ = 81 min [75 – 89 min]. At 0C, the dissociation was slowed to $t_{1/2\ Fast}$ = 14 min [10 – 23 min] and $t_{1/2\ Slow}$ = 244 min [207 – 299 min].

More detailed dissociation studies were undertaken for [^{125}I]-6 (Figure 45, revealing the presence of both fast and slowly dissociating components with halflives of dissociation of $t_{1/2\ Fast}$ = 4.6 min [4.1 – 5.2 min], $t_{1/2\ Slow}$ = 81 min [75 – 89 min] respectively at room temperature, which slowed markedly to $t_{1/2\ Fast}$ = 14 min [10 – 23 min] and $t_{1/2\ Slow}$ = 244 min [207 – 299 min] in an ice-water bath. As high affinity binding was abolished by GppNHp and sodium, the slowly dissociating site is consistent with binding to the agonist conformation of the mu receptor while the quickly dissociating site appears to correspond to binding of the antagonist conformation of the receptor.

After extensive optimization, protocols for photoaffinity labeling different tissues were developed. First efforts focused on mu receptors stably expressed by the CHO-MOR cell line (Figure 46A, Figure 47A). Following photolabeling, the labeled species ran as a diffuse band of approximately 68-75 kDa, consistent with the molecular weight of the mature, glycosylated form of the murine mu opioid receptor expressed on the cell surface, with no bands observed at the molecular weight of the unglycosylated, immature forms of the receptor, demonstrating one significant advantage in detection and quantitation of the photolabels. Levallorphan blocked receptor labeling, further confirming the identity of the photolabeled species and western blot with an MOR antibody confirmed that mu opioid receptor protein was present in both lanes (Figure 47B). Furthermore, cleavage of N-linked sugars from the receptor with PNGaseF yielded a photolabeled species with the predicted molecular weight of the mu receptor's amino acid sequence alone, 44kDa (Figure 47C).

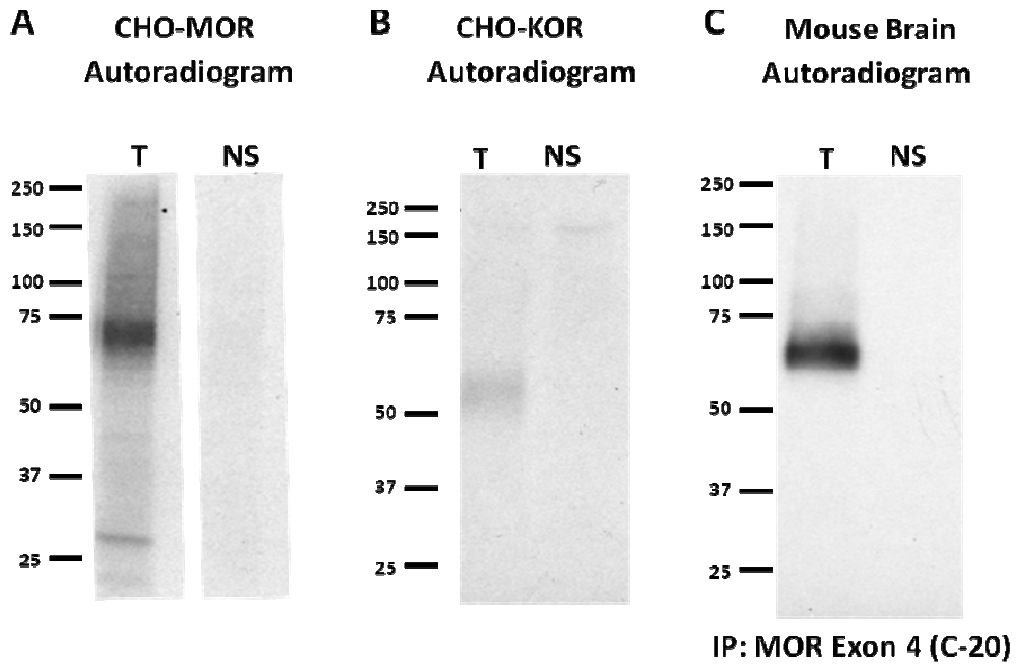
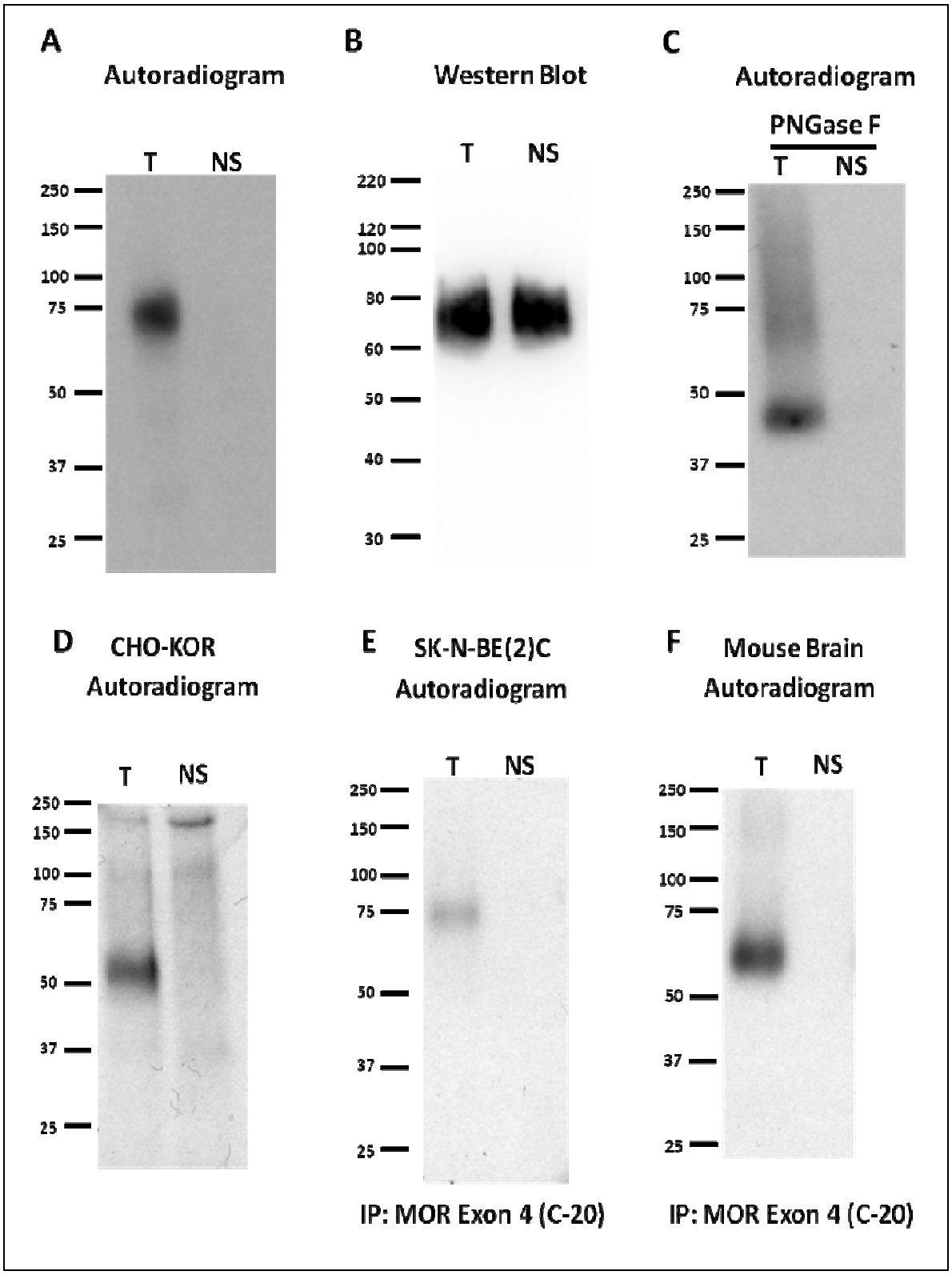


Figure 46. Photoaffinity labeling with [^{125}I]-6 in CHO-MOR, CHO-KOR, and Mouse Brain.

The indicated tissues were photolabeled with [^{125}I]-6 and separated by SDS-PAGE. All autoradiograms are representative and have been independently replicated at least twice. T, Total; NS, Nonspecific. (A) CHO-MOR. The photolabeled species ran as a diffuse band of approximately 68-75 kDa, consistent with the molecular weight of the mature, glycosylated form of the murine mu opioid receptor. (B) CHO-KOR. KOR was enriched by wheat germ affinity purification. The photolabeled species ran as a diffuse band of approximately 50-57 kDa, consistent with the mature, glycosylated form of murine kappa opioid receptor. (C) CD1 Mouse Brain. Mu opioid receptors in mouse brain were photolabeled and purified by immunoprecipitation with an antibody to the C-terminal region of the receptor. running with lower apparent molecular weight than observed in cell lines, approximately 60-65 kDa.

Figure 47. Photoaffinity labeling with [^{125}I]-7 in CHO-MOR, CHO-KOR, SK-N-BE(2)C, and Mouse Brain.

The indicated tissues were photolabeled with [^{125}I]-6 and separated by SDS-PAGE. All autoradiograms are representative and have been independently replicated at least twice. T, Total; NS, Nonspecific. (A) CHO-MOR Photolabeled mu receptors ran as a diffuse band of approximately 68-75 kDa. (B) MOR Western Blot. Probing membrane from (A) with MOR antibody confirmed the presence of mu receptors in both Total and Nonspecific lanes. (C) PNGaseF. Deglycosylation with PNGase F reduced the molecular weight to the predicted molecular weight of 44 kDa. (D) CHO-KOR. Following Wheat Germ affinity enrichment, photolabeled kappa opioid receptors ran as a diffuse band from 50-57 kDa. (E) SK-N-BE(2)C neuroblastoma. Mu opioid receptors were photolabeled in a natively-expressing cell line, appearing as a diffuse band from about 70-77 kDa. (F) C57 Mouse Brain. Mu receptors in mouse brain were ran with a lower apparent molecular weight than observed in cell lines, approximately 57-65 kDa.


A
Autoradiogram
T
NS
250
150
100
75
50
37
25
B
Western Blot
T
NS
220
120
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80
60
50
40
30
C
Autoradiogram
PNGase F
T
NS
250
150
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75
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37
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SK-N-BE(2)C
Autoradiogram
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250
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37
25
IP: MOR Exon 4 (C-20)
F
Mouse Brain
Autoradiogram
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NS
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25
IP: MOR Exon 4 (C-20)

CHO-KOR could also be photolabeled although much less efficiently, resulting in a poorer ratio of total to nonspecific labeling. Photolabeled kappa receptors ran with a rough peak at the expected 55kDa (not shown) but could be more cleanly observed following wheat germ affinity chromatography to isolate glycosylated receptor, running as a diffuse band from about 50-57kDa (Figure 46B, Figure 47D).

After optimization with highly overexpressing cell lines, we next attempted to photoaffinity label opioid receptors in natively expressing tissues. Although nonspecific labeling in mouse brain initially prevented visualization of receptors, the addition of an immunoaffinity enrichment step dramatically improved detection. CD1 (Figure 46C) or C57 (Figure 47F) mouse brain membranes showed a distinctly lower molecular molecular weight band for mu receptors, although the this difference in glycosylation state between cell lines and even different brain regions has been demonstrated previously (Peng Huang et al., 2008). Indeed, even SK-N-BE(2)C membranes, a human neuroblastoma cell line which natively expresses mu opioid receptors and splice variants, showed a higher molecular weight, much closer to that seen in heterologously expressing cell lines (Figure 47E).

We next attempted to use the technique of blue native PAGE, pioneered by Schägger & von Jagow (1991) as a modification of traditional native PAGE to permit estimations of molecular weight of proteins with their quaternary interactions unperturbed. Photolabeled and immunoprecipitated MOR ran as a single diffuse band at an approximately 200kDA in both CHO-MOR and mouse brain membranes (Figure 48). Further separation by the addition of a second dimension of electrophoresis showed that this band migrated at the expected 70-75 kDa under denaturing and reducing conditions.

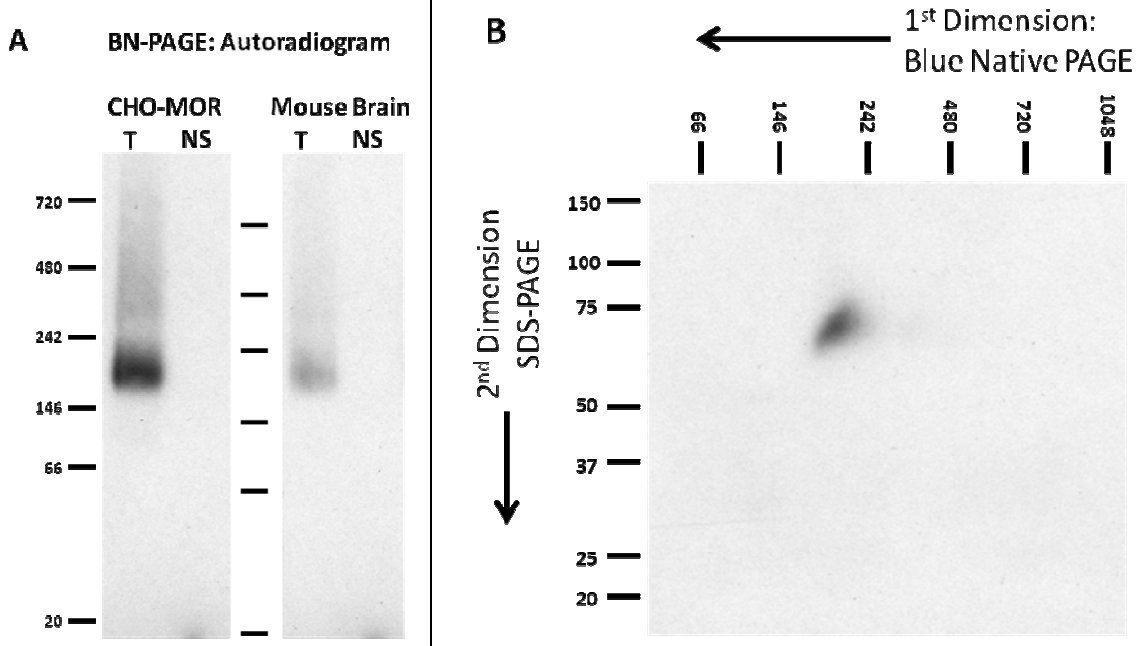

Figure 48. BN-PAGE and 2D BN-PAGE/SDS-PAGE of [^{125}I]-6 Photolabeled Mu Opioid Receptors.
(A) CHO-MOR and Mouse brain membranes were photolabeled and immunoprecipitate under nondenaturing conditions before separation by blue native PAGE to yield a single diffuse band with an approximate molecular weight of 200kDa. (B) CHO-MOR. A lane from a BN-PAGE gel run on photolabeled CHO-MOR membranes was excised from a BN-PAGE gel, denatured and reduced, and then run in an orthogonal direction on an SDS-PAGE gel to give 2 dimensions of separation. The 200kDa band from the BN-PAGE gel ran at 70-75 kDa, consistent with previous observations for the mu receptor. Results are representative figures and have each been replicated by at least 2 independent experiments.

Finally, we achieved the intended purpose of these ligands when an unidentified target labeled by [^{125}I]-7 was successfully photolabeled, enriched by immunoprecipitation by C-terminal MOR antibody, and visualized following SDS-PAGE separation. This is hypothesized to be the truncated 6TM splice variant that is the target for IBNtxA and presumably mediates its analgesic actions independently of the traditional mu, delta, and kappa opioid receptors.

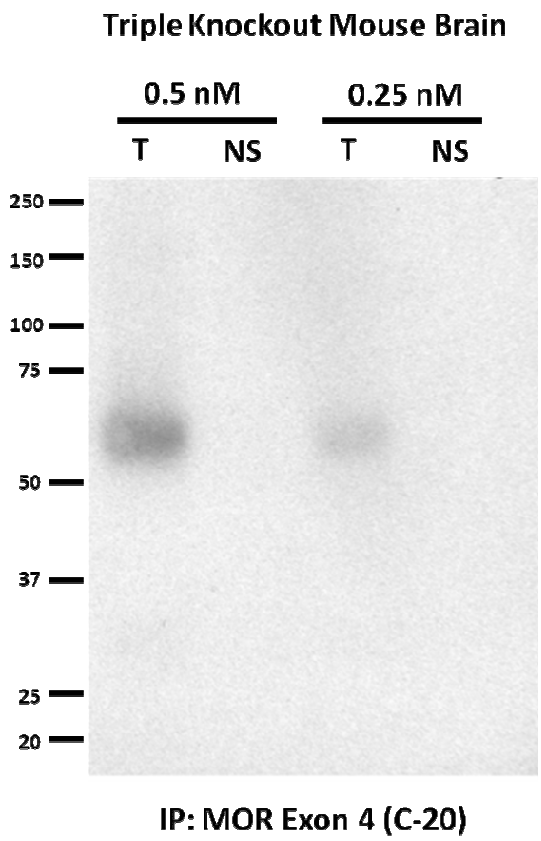

Figure 49. Photolabeled Target in Triple Knockout Mouse Brain.
[^{125}I]-7 was used to photolabel the unknown target of IBNtxA in triple knockout mouse brain, which was enriched by MOR exon 4 immunoprecipitation. No further tissue from this animal could be obtained for a replicate so results should be interpreted with caution accordingly.

Discussion

Photoaffinity labeling offers a way to connect the realm of pharmacologically defined receptors with their molecular identities. It also permits separation of receptor binding on the basis of size or other chromatographic attributes, offering a convenient tag for the development of enrichment steps and complex purification schemes. Here, we synthesized 2 new photoaffinity labels based on the structure of IBNalA for

photolabeling mu and kappa sites, in addition to as-yet unidentified receptors such as those observed in triple knockout mouse brain.

Both photolabels demonstrated very high affinity for mu and kappa opioid receptors, with moderate affinity for delta receptors. The change of the N-substituent from an allyl (6) to a propargyl group (7) resulted in a minor 2-fold loss of affinity for mu opioid receptors but a >4-fold increase in affinity for the "6TM/E11" target of IBNtxA in triple knockout mouse brain, suggesting that this compound in particular might aid in the identification of the target and its components.

Radioiodinated forms of the drug were synthesized and found to have even higher affinity for mu and kappa receptors than predicted based on competition studies. Consistent with the extremely high affinity exhibited for mu opioid receptors, the allyl photoaffinity label (6) displayed a halflife of dissociation greater than 1 hour at room temperature and greater than 4 hours in an ice bath even before covalent coupling of the drug to the receptor. This extremely high affinity aided visualization efforts by substantially increasing the ratio of total to nonspecific binding. Although substantial nonspecific labeling was observed in natively expressing tissues, the addition of an immunoprecipitation enrichment step enabled visualization of mu opioid receptors in natively expressing neuroblastoma and mouse brain membranes.

Interestingly, mu receptors migrated with a markedly different apparent molecular weight in mouse brain tissue relative to that observed in both heterologously and natively expressing cell lines. This appears to be due to different glycosylation patterns, previously reported by the Liu-Chen lab (Peng Huang et al., 2008). In fact, Huang et al found different apparent molecular weights for mu receptors labeled by an antibody directed at the protein's C-terminal tail even between brain regions, with receptor from the mouse striatum running from 60 – 84 kDa (median 74 kDa), while

receptor from mouse thalamus ran with a similar narrower, lower molecular weight as we observed, from 58 – 68 kDa (median 63 kDa). Their findings were similar in rat striatal and thalamic membranes, suggesting a similar pattern of glycosylation at least across rodent species. Thus, it appears that at least on the basis of size, our cell lines express a more "striatal-like" glycosylation pattern, while the majority of mu receptors in whole mouse brain homogenate appear to express a more "thalamic" pattern, consistent with the extremely high density of receptor there. It is unclear what ramifications this might have for the use of heterologously-expressing and neuroblastoma cell lines as model systems for the study of *in vivo* opioid pharmacology. These results present another reminder that models should never be mistaken for nature itself and must always be used with limitations in mind.

In addition to separation by SDS-PAGE, we resolved opioid receptors and their complexes using non-denaturing blue native gel electrophoresis. On BN-PAGE gels, the photolabeled mu receptor species appeared to migrate at a rough molecular weight of 200 kDa for both CHO-MOR and mouse brain. Several cautions must be addressed regarding this rough estimate: first, BN-PAGE is less accurate as an estimate of molecular weight than SDS-PAGE. Rather than the denaturing SDS, Coomassie brilliant blue G-250 dye is used as a non-denaturing charge shift molecule to ensure that all proteins move toward the anode proportionally to the quantity of dye bound to their negatively charged residues, approximately proportionally to the protein's molecular weight. However, there may be some nonlinearities introduced by the migration of multiprotein complexes especially of multiple oligomeric states, and differing detergents. We did not observe a difference in molecular weight for differing detergents (MNG-3, DDM, CHAPS); in fact, even protein solubilized in urea and SDS and reduced with dithiothreitol ran with the same apparent molecular weight (not shown). These

results suggesting that the species observed is may not be a true signaling complex as initially hoped, but could reflect the tendency of receptor to form oligomers under non-denaturing conditions as noted by Simonds et al following purification of delta receptors (Simonds et al., 1985).

However, although this may make the BN-PAGE less useful from an analytical standpoint, this technique can still be used preparatively to provide a second dimension of separation for complex mixtures of proteins. This may be especially useful in future attempts for unknown receptors for which wheat germ or immunoaffinity enrichment steps are not possible or are too low yielding to be of use.

Finally, we present the first information regarding the target of IBNtxA in triple knockout mouse brain. Although a single experiment, the findings were consistent with the labeling of a truncated 6TM variant postulated to mediate the actions of IBNtxA and offers insights into future studies of this target.

Chapter 7: Discussion

Following their discovery in 2001, truncated 6TM splice variants of the mu opioid receptor were considered a curiosity that had little physiological relevance as they failed to bind drug on their own and were expressed at low levels relative to traditional mu opioid receptors. The demonstration in 2009 that the loss of MOR exon 11-associated splice variants resulted in dramatically lower potency of heroin, M6G, and fentanyl, but not morphine or methadone, suggested that they might have a more important role after all. Here, we have demonstrated a critical role for this set of splice variants in mediating the analgesia of multiple classes of drugs, both new and old, opioid and non-opioid, independent of many dose- and use-limiting side effects.

The synthesis of the powerful and unique new pain reliever, IBNtxA, conclusively implicated 6TM receptors associated with MOR exon 11 in its mechanism of action. Importantly, despite a non-selective binding profile, IBNtxA's analgesia was not siginificantly affected by loss of delta, kappa, and exon 1-associated MOR splice variants, suggesting that the remaining 6TM receptors were a target for powerful analgesia. More importantly still, IBNtxA's side effect profile showed improved or absent side effects classically associated with mu opioid agonists such as morphine. In mice, IBNtxA produced no respiratory depression, slow development of tolerance, no apparent dependence syndrome, mild constipation (off-target), and most importantly no reward in a conditioned place preference assay. In rats, IBNtxA was slightly less potent but showed no cross-tolerance with morphine and also did not produce respiratory depression. Like behavior, [^{125}I]-IBNtxA binding in rat brain membranes showed similar pharmacological trends as in mice although heterogeneity was observed, suggesting the possibility of more than one target. Studies of the target's regional distribution revealed highest levels in the thalamus, with binding virtually absent in the cerebellum.

Since the studies herein were published, IBNtxA's efficacy in other pain models of neuropathic and inflammatory pain have stoked further interest in the target (Wieskopf et al., 2014). Additionally, the recent demonstration by Lu et al that lentiviral restoration of mMOR-1G - a 6TM isoform present in the mouse, rat, and human - can rescue IBNtxA analgesia in an Exon 1/Exon 11 total mu knockout animal, puts the physiological role of 6TM receptors beyond doubt (Lu et al., 2015).

Next, we re-examined the pharmacology of another historically unusual opioid, buprenorphine, which shared many surface similarities with IBNtxA. To our surprise, buprenorphine analgesia was totally lost in MOR exon 11 knockout animals, while animals lacking MOR exon 1 variants still retained ~40% analgesic efficacy. Lentiviral restoration of mMOR-1G expression in MOR Exon 11, but not Exon 1/11 knockout mice, rescued buprenorphine analgesia, confirming the involvement of both 6TM and 7TM receptor isoforms.

Side effects present in buprenorphine treated animals such as locomotor stimulation and inhibition of gastrointestinal transit were unaffected in MOR exon 11 knockout animals, suggesting that these undesireable effects have a distinct mechanism of action from the target mediating the desired analgesic effects of the drug. In vitro assays showed buprenorphine to be an agonist at mu receptors, weak partial agonist or neutral antagonist at delta receptors, and inverse agonist at kappa receptors, although studies in cell models could not be followed up in brain tissue as no stimulation could be observed in brain. Finally, we confirmed that buprenorphine is a perfect biased agonist at mu opioid receptors, failing to recruit β-arrestin even at 10μM and potently antagonizing the β-arrestin recruitment of DAMGO.

We next looked at kappa$_1$, alpha$_2$ adrenergic, and cannabinoid type 1 agonist analgesia and found that kappa$_1$ and alpha$_2$ adrenergic analgesia was significantly

impaired, while cannabinoid analgesia was entirely intact. As with buprenorphine, lentiviral restoration of mMOR-1G in 2 different knockout models rescued analgesic efficacy of kappa$_1$ and alpha$_2$ adrenergic agonists. However, as with buprenorphine, side effects – for kappa$_1$ agonists, dysphoria; for alpha$_2$ agonists, sedation as measured by depression of spontaneous locomotor activity – were retained in MOR exon 11 knockout animals, genetically dissociating these effects from the desired analgesic effect and suggesting that drugs from these classes might be possible next-generation analgesics, devoid of side effects which have thus far limited their more widespread use.

Finally, 2 new photoaffinity label compounds were synthesized and used to photolabel opioid receptors as an attempt at bridging the gap between the world of pharmacologically defined receptor targets and the genetic identity of the macromolecules binding and mediating the effects of those drugs. These compounds are further useful as tags for the quantitation and development of purification schemes with known mu opioid receptor splice variants and their associated protein ensembles. Protocols were developed for receptors expressed in cell lines and enrichment schemes based on immunoaffinity and glycosylation status facilitated the visualization of less abundant receptors in natively expressing tissues such as SK-N-BE(2)C and mouse brain which both express multiple MOR-1 splice variants. Additionally, we present, to our knowledge, the first examination of MOR migration on BN-PAGE and 2D-BN-PAGE/SDS-PAGE, where the receptor migrated with an apparent molecular weight of 200 kDa, much greater than that of its monomeric, glycosylated form.

Finally, we present initial information regarding the target of IBNtxA in triple knockout mouse brain, although further work is needed to confirm and extend these observations. Current efforts are ongoing with other triple knockout mouse brain tissue as well as other tissues believed to express the target mediating IBNtxA's effects.

The overarching question is, of course, how do these truncated 6TM isoforms function, if they do not bind drug on their own? One critical observation is that in transfected cell lines, 6TMs appear to be retained in the endoplasmic reticulum rather than being expressed at the cell surface (unpublished observations) – however, in MOR Exon 1/Exon 11 knockout animals with lentiviral rescue of MOR-1G expression, receptor was observed at the cell surface (Lu et al., 2015). As the loss of the normal extracellular N-terminal domain from exon 1 results in a loss of all predicted glycosylation sites of the receptor, alterations in trafficking are not surprising, as normal chaperones which ensure proper folding and transport may require glycosylation. One possibility is that some other protein acts as a chaperone *in vivo* which is absent from the heterologously-expressing cell lines. If this is true, our current characterization of 6TMs as failing to bind drug may even be incorrect.

Future experiments will also be necessary to determine whether MOR-1G is the particular 6TM which is involved or whether other 6TMs, such as MOR-1M and MOR-1N which differ at the C-terminus can also rescue analgesic response to IBNtxA, buprenorphine, kappa$_1$, and alpha$_2$ adrenergic agonists.

In conclusion, we have revealed multiple new 6TM-associated targets for the development of new pain relievers with superior side effect profiles. These results show that the concept of receptor multiplicity is alive and well, and that careful research can reveal differences in what previously appeared to be homogenous populations of receptors.

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