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Medications development for drug addiction and other neuropsychiatric disorders

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**MEDICATIONS DEVELOPMENT FOR DRUG ADDICTION AND OTHER
NEUROPSYCHIATRIC DISORDERS**

by
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A Thesis

Department of Chemistry & Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
Rowan University
August 13, 2018

Thesis Advisor: Thomas M. Keck, Ph.D.

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Dedications

Dedicated for my parents Aleya Begum and Sirajul Islam who always beside me and keep believing in me.

Acknowledgments

I would like to acknowledge my family and friends, who have been providing me continuous support for my education. I would also like to thank all collaborators and my research group. Finally, I will be always thankful to my research advisor for the time he has spent to educate me, the patience for listening to my repeated questions, and to provide me the opportunities to learn and explore novel things which were never limited only in Rowan. Not only is he a great mentor, he has been a wonderful friend to me.

Abstract

Ariful Islam
MEDICATIONS DEVELOPMENT FOR DRUG ADDICTION AND OTHER
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2018-2019
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Master of Science in Pharmaceutical Science

Drug addiction and abuse especially opiate and psychostimulant abuse is a national and global crisis. IBNtxA (3-iodobenzoyl naltrexamine) is a novel μ opioid receptor (MOR) agonist, a naloxone derivative, structurally related to the classical MOR antagonist naltrexone. Recent studies suggest IBNtxA preferentially signals through truncated MOR splice variants, producing a unique pharmacological profile resulting in potent analgesia with reduced side effects. It has been found that *M. vaccae* has immunoregulatory effects that can prevent stress-induced exaggeration of neuroinflammation in the brain. The purpose of our pilot study is to develop medication for addiction and neuropsychiatric disorders. According to our purpose, we evaluated a range of IBNtxA doses to more fully assess its abuse liability and antiaddiction properties and the preimmunization effect of heat-killed *M. vaccae* on cocaine addiction. IBNtxA represents an intriguing lead compound for preclinical drug development specifically targeting MOR splice variants, potentially creating effective analgesics with reduced side effects. Furthermore, IBNtxA could have use as an adjunct therapy in agonist replacement strategies (e.g., methadone). *M. vaccae* might be helpful for cocaine relapse. Current collaborative efforts are aimed to find the total signaling pathways of IBNtxA and the effect of *M. vaccae* on cocaine self-administration, cocaine induced neuroinflammations and to keep finding medicine for neurological diseases.

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Chapter 1

Introduction

Background

A brief history of opiate drugs. Opiates are a very old class of analgesic drugs derived directly from the opium poppy. (Manglik et al., 2012)The term opiate refers specifically to [list compounds]. Since opium has a long history, it is pretty difficult to claim when it was first used (or abused), but scholars agree that the Sumerians in Mesopotamia, present-day Iraq, cultivated and isolated opium from the opium poppy (*Papaver somniferu*) around 3400 B.C. They named the plant “hul gil,” meaning “the plant of joy.”(Brownstein, 1993; Rosenblum, Marsch, Joseph, & Portenoy, 2008) In 1806, the German chemist Friedrich Wilhelm Adam Sertürner isolated morphine from opium and named it “morphine” after the god of dreams, Morpheus. (Brownstein, 1993; Duarte, 2005) In the 1850s, Alexander Wood reported that he had injected morphine into his wife as an experiment, using his invented syringe with hollow needle, and his wife died from respiratory depression. (Jonkman et al., 2018)

In the United States, opiates like morphine have been used since the American Civil War as potent analgesics. Opiate use outside of medical treatment led to opiate abuse and addiction. The Harrison Narcotics Tax Act was passed in 1914 to curb the addiction and abuse of some highly addictive opiates and cocaine, which means possession without a prescription of these drugs inside the United States is a criminal offense. (Terry, 1915)

The term opioid means opiate-like—a combination of the word opium and the suffix –oid, meaning “like” or “resembling”—originated in 1950, and was first proposed by Dr. George H. Acheson. (Wikler, Martin, Pescor, & Eades, 1963) Opioid drugs have

structural similarities with morphine but are either synthetic or semisynthetic. (Martin, 1983) The endogenous (i.e., naturally occurring) opioid peptides, endorphins, were first discovered in 1974 by two independent group of investigators—John Hughes and Hans Kosterlitz of Scotland, and Rabi Simantov and Solomon H. Snyder of the United States. (McLaughlin & Zagon, 2013)

Opioid drug classifications. Opioid drugs encompass a broad spectrum of activity. According to their synthetic process, clinical opioids can be classified into three groups:

1. Naturally obtained, extracted directly from poppy seeds, such as morphine, papaverine, and codeine;
2. Semi-synthetic compounds, which feature modifications of natural compounds, including morphine esters such as heroin, oxycodone, and oxymorphone;
3. Fully synthetic compounds, such as pethidine, fentanyl, and tramadol. (Jamison & Mao, 2015; Pathan & Williams, 2012)

Opioids can also be classified based upon their binding affinity and effects on the four major opioid receptors:

1. The δ -opioid receptor (DOR);
2. The κ -opioid receptor (KOR);
3. The μ -opioid receptor (MOR);
4. The nociception/orphanin opioid receptor (NOR).

Finally, opioids can be classified based on their signaling properties:

1. Full agonists (e.g., morphine, etorphine, methadone, meperidine, codeine, hydromorphone etc.), which will fully activate a given opioid receptor;

2. Partial agonists (e.g., buprenorphine, pentazocine, nalbuphine), which will partially activate a given opioid receptor;
3. Antagonists (e.g., naloxone, naltrexone insert other examples?), which will block the activity of agonists or partial agonists. (Jamison & Mao, 2015; Waldhoer, Bartlett, & Whistler, 2004)

Other than clinical opioids, the body itself also produces opioid peptides, commonly known as endogenous opioids or endogenous ligands. These endogenous opioids bind to opioid receptors and exert pharmacological actions. (Holden; Li et al., 2012; Waldhoer et al., 2004) Though there are many identified endogenous opioid peptides, they can be classified into three groups of ligands—enkephalins, endorphins, and dynorphins—which generally signal through the three major receptors, DOR, MOR, and KOR, respectively. (Li et al., 2012)

Table 1

Common clinical and endogenous opioid ligands. (Egan, 2005; Endoh, Matsuura, Tanaka, & Nagase, 1992; Gilman, 2011; Jamison & Mao, 2015; Maisonneuve, Archer, & Glick, 1994; Pathan & Williams, 2012; Trescot, Datta, Lee, & Hansen, 2008)

Opioid Ligands	Mu Opioid Receptor	Delta Opioid Receptor	Kappa Opioid Receptor	Nociceptin Receptor
<i>Endogenous Ligands</i>				
β -endorphin	+++	+++		
Enkephalins	++	+++		
Dynorphin A&B	++	+	+++	+
Nociceptin/orphanin FQ				+++
<i>Clinical and Nonclinical Ligands</i>				
<i>Agonists</i>				
Morphine	+++		+	
Codeine	+	+	+	
Fentanyl	+++	+		
Pethidine	+++	+	+	
Methadone	+++			
U50,488			+++	
TAN-67 (SB-205,607)		+++		
<i>Partial Agonists</i>				
Buprenorphine	+		--	
Pentazocine	-	+	++	
<i>Antagonists</i>				
Naloxone	---	-	--	
Naltrexone	---	-	---	
Nor-binaltorphimine			---	

(+) sign indicates receptor selectivity of opioid agonists, more (+) sign means more selectivity, no (+) sign indicates, no selectivity. (-) sign indicates receptor selectivity of opioid antagonists, more (-) sign indicates more antagonist effect.

Opioid receptors. Opioid ligands signal through opioid receptors, which are members of the 7-transmembrane G protein-coupled receptor (GPCR) superfamily. Opioid receptors are widely found in the central nervous system but also found in the peripheral nervous system. (Stein, 2016; Waldhoer et al., 2004) Though these receptors belong to the same class, their functions and cellular localizations are quite different. Most full agonists, such as morphine, endomorphins, fentanyl, and pethidine, primarily show pharmacological effects upon binding through MOR. (Stein, 2016; Trescot et al., 2008) It has three major subtypes of MOR, μ_1 , μ_2 , and μ_3 , located (*Figure 2*) in the brain—primarily in the cortex, thalamus, periaqueductal gray (PG)—and spinal cord substantia gelatinosa. MOR are also heavily expressed in the intestinal tract. (Mao, 1999; Stein, Schäfer, & Machelska, 2003) Individual MOR subtypes have different functions: μ_1 is responsible for supraspinal analgesia, and physical dependence; μ_2 is responsible for respiratory depression, euphoria, physical dependence, reduced gastrointestinal motility, and miosis; and μ_3 may affect vasodilation. (Mao, 1999; Stein et al., 2003)

Endogenous opioids, like endorphin and enkephalin, have more receptor selectivity for DOR over clinical opioids which are most available pontine nuclei, amygdala and olfactory bulbs of CNS (*Figure 2*). It has two subtypes δ_1 & δ_2 , though individual subtype's function is not so obvious but DOR responsible for analgesia, euphoria, physical dependence, convulsant, and antidepressant effects. (Chung & Kieffer, 2013; Mao, 1999) In the brain, KOR mostly presents in hypothalamus, periaqueductal gray, and claustrum; in the spinal cord, KOR mostly presents in the substantia gelatinosa (*Figure 2*). KOR activation produces spinal analgesia, sedation, miosis, dysphoria, neuroprotection, and diuresis. KOR has three known subtypes, κ_1 , κ_2 , and κ_3 . (Lalanne, Ayranci, Kieffer, & Lutz,

2014; Stein et al., 2003) NOR is densely expressed (*Figure 2*) in cortex, ventral forebrain, hippocampus, hypothalamus, amygdala, and in the dorsal horn of spinal cord. (Donica, Awwad, Thakker, & Standifer, 2013; Koob, Arends, & Le Moal, 2014) Activation of NOR, produces some physiological pharmacological responses such as anxiety, food intake, learning, locomotor etc. (Donica et al., 2013)

Mechanisms action of opioid agonists. Opioids bind to opioid receptors, producing a series of intracellular changes resulting in pharmacological effects. First, GTP binding at $G\alpha$ subunit, guanosine triphosphate (GTP) exchanges to guanosine diphosphate (GDP) that triggers α -GTP complex to dissociate away from the $\beta\gamma$ complex (*Figure 1*). (McDonald & Lambert, 2005; Pathan & Williams, 2012; Stein, 2016) Free α -GTP and $\beta\gamma$ interact with the target protein and inhibit adenylate cyclase, which decreases cyclic adenosine monophosphate (cAMP) inside the cell (*Figure 2*). (McDonald & Lambert, 2005; Pathan & Williams, 2012) MOR, DOR, and KOR signaling can also moderate Ca^{2+} channels (*Figure 1*) in both pre- and post-synapse reduces Ca^{2+} inside cell and impaired the neurons' excitability. (Simons, 1988; Stein, 2016) These intercellular events cause hyperpolarization as well as hinder neuronal firing in key nociceptive circuits and eventually reduces pain. (McDonald & Lambert, 2005; Pathan & Williams, 2012; Simons, 1988; Stein, 2016)

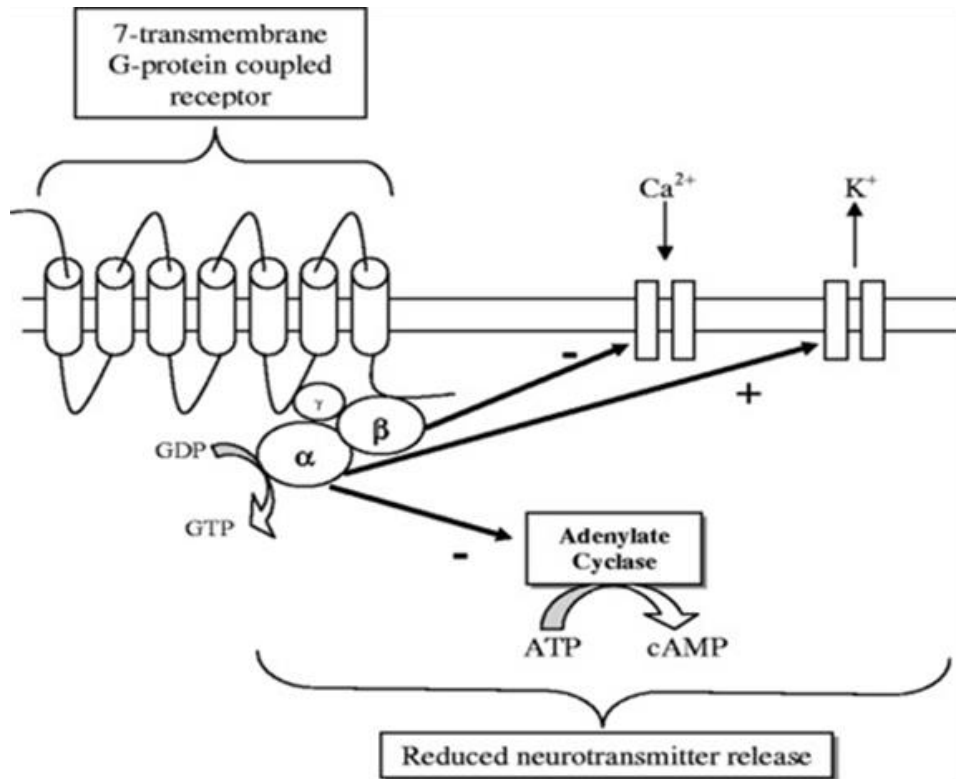


Figure 1. from McDonald and Lambert (2005) Mechanism of actions opioid receptor ligands. Binding of an opioid agonist to a G protein-coupled opioid receptor induces the G α protein to exchange its bound GDP for GTP, which causes G α -GTP complex to dissociate away from the G $\beta\gamma$ complex, and all G proteins to dissociate from the receptor. (Pathan & Williams, 2012) Free G α -GTP and G $\beta\gamma$ interact with target proteins. $\alpha_{i/o}$, the G protein associated with all opioid receptors, inhibits adenylate cyclase, reducing synthesis of cAMP. G α and G $\beta\gamma$ also have complex interactions at various Ca $^{2+}$ and K $^{+}$ ion channels. In neurons, opioid receptor activation typically results in the suppression of neuronal firing. (McDonald & Lambert, 2005)

Extensive use of opioid drugs.

Pain management. Over a hundred years, opioids have been used for treating both chronic and acute pain which is not only the national health issue but also an important global health issue. In the United States, more than 100 million people suffer from chronic pain and among them, 5-8 million use opioid drugs for long-term treatment. (Jamison & Mao, 2015; Kalso, Edwards, Moore, & McQuay, 2004) Opioid analgesics are very effective in both cancer and non-cancer pain too. Several clinical studies showed that intravenous infusion of opioid analgesic significantly reduces the neuropathic pain like central pain, postherpetic neuralgia, mixed neuropathic pain. (Kalso et al., 2004) Numerous studies had confirmed the significance of different doses of oral opioid analgesics on neuropathic, musculoskeletal and other non-cancer pain. (Kalso et al., 2004) The effectiveness of opioid drugs to manage cancer pain has already been confirmed by WHO. Severe pain in cancer patients is very challenging to manage and almost 75% of them need to be treated with opioids analgesics. (Thapa, Rastogi, & Ahuja, 2011) Among all opioid drugs, morphine alone is sufficient for 85% of patients as single pharmacotherapy to treat severe cancer pain. In other cases, combination therapy with other analgesics or anti-neoplastic drugs can be more effective. (Gilson, Ryan, Joranson, & Dahl, 2004) Sometimes, switching from one opioid drug (which already has receptor-resistance) to another opioid drug can be effective to get superior analgesic effect. (Thapa et al., 2011) Though success rate of opioid drugs is higher in cancer pain management but there are many barriers make it intricate. Unavailability of morphine, economic crises in developing countries, and improper pain assessment are some of the obstacles for pain management. (Thapa et al., 2011)

Acute pulmonary edema. Morphine has been used for a long time to treat pulmonary edema. Because of the failure of the left ventricle, the pulmonary circulation also increases due to elevated hydrostatic pressure which causes extra fluids accumulation into interstitium and alveoli of lungs. (Ellingsrud & Agewall, 2016) The aim of pulmonary edema treatment is to reduce hydrostatic pressure through lowering preload and afterload that can be achieved by vasodilation. (Mattu, Martinez, & Kelly, 2005) It has been thought, though not obvious, morphine has both anxiolytic and vasodilatory properties which can treat elevated pulmonary fluids by dilation.(Ellingsrud & Agewall, 2016) The European heart failure guideline considered intravenous infusion of morphine 4-8 mg as treatment as a treatment of pulmonary oedema guideline in 2012 through the American College of Cardiology Foundation/American Heart Association did not consider morphine for treating this disorder as a treatment guideline. (McMurray et al., 2012; Yancy et al., 2013) However, the American Heart Failure Society suggests if morphine needs to be administered then should be incautious. ("Section 12: Evaluation and Management of Patients with Acute Decompensated Heart Failure," 2010)

Diarrhea. Interestingly opioid drugs can treat irritable bowel syndrome with diarrhea (IBS-D). Since there are limited options available for managing IBS-D, so finding new treatment methods is crucial. Investigations on phase-3 clinical trials have found a substantial effect of eluxadoline, a new oral mu opioid receptor agonist but it has mixed opioids effect such as it also agonist KOR but antagonist of DOR, on IBS-D. (Lembo et al., 2016)

Cough. Codeine has been used for decades as an antitussive opioid medication for cough treatment. Numerous recent studies question the efficacy of codeine in treatment of

cough. One study found that codeine did not have any significant effect on chronic obstructive pulmonary disease (COPD) when compared to placebo even though it is a standard antitussive agent. (Smith, Owen, Earis, & Woodcock, 2006) Codeine is ineffective as a cough suppressant when it is generated due to upper respiratory disorder or COPD, and it is not even effective for an acute cough in children. The U.S. Food and Drug Administration (FDA) recently warned that any cough medication containing opioids should not be used by patients less than 18 years old. However, still few studies claim, codeine is effective for a chronic cough in adults. (McCrorry et al., 2013)

Anesthesia. Opioid analgesics, especially narcotic opioids, have been widely using as anesthetic agents for various terms of anesthetic medications practice. Opiates analgesics are commonly used for major surgeries most specifically the surgical operation of any patient who has the cardiovascular disorder. (Bovill, Sebel, & Stanley, 1984) The principal reason for using opioids in patients with the cardiovascular disease during surgery is the absence of cardiac depression. (Hug, 1992) Though opioid medications have some side-effects in comparison with other anesthetic agents narcotic opioids even many cases better. (Bovill et al., 1984; Hug, 1992) Proper knowledge of opioids pharmacology, dose-response curves, and possible adverse effects can easily help to manage limitations of narcotic opioid analgesics as anesthesia. (Hug, 1992)

Adverse Effects of Opioids

Opioids analgesics accompany with inevitable adverse effects including respiratory depression, sedation, euphoria, constipation, bradycardia etc. (Ballantyne & Mao 2003; DeWire et al., 2013) Along with side effects, other adverse effects such as the addiction, abuse, dependency, tolerance, hyperalgesia, and withdrawals symptoms make opioids analgesic difficult to deal. (Fields & Margolis, 2015; Jamison & Mao, 2015; Volkow & McLellan, 2016)

Tolerance. The term drug tolerance or medication tolerance can be simply defined as when the body doesn't response or show same pharmacological actions as it shows initially with the same drug which means body requires higher dose over time to demonstrate the desired therapeutic effect.(Savage et al., 2003) When drug tolerance happens due to repeated opioids drugs exposure, the term could be renamed—opioid tolerance.(Chang, Chen, & Mao, 2007) Scientists had been investigating the mechanism behind this opioid analgesic tolerance and found multiple mechanisms but the most convincing mechanisms are opioid receptor desensitization and internalization.(Pan, 2007) Desensitized receptors decrease their functions by limiting cellular signaling and internalization causes receptors reduction on the surface of the cell membrane and decreases receptors availability for opioids ligand bindings. (Allouche, Noble, & Marie, 2014; Pan, 2007) This signal transduction event takes place by two steps: the first step of desensitization occurs through GPCR phosphorylation by G protein-coupled receptor kinases (GRKs) which makes receptors ready for the second step—arresting bindings. (Pan, 2007) Once arresting binding happens, it eventuates uncoupling G protein signaling from GPCRs through blockage of G protein binding and other than only develops desensitization, it also

promotes internalization. (Krupnick, Goodman, Keen, & Benovic, 1997) Switching to another opioid analgesic or in combination with other analgesics as a part of pain therapy helps to bypass opioid tolerance but probably finding complete new opioid agonist could be more helpful.

Hyperalgesia. Opioid-induced hyperalgesia (OIH) is the state of a paradoxical nociceptive sensitization response due to long-term opioids exposure. (Lee, Silverman, Hansen, Patel, & Manchikanti, 2011) When it is supposed to reduce pain after getting opioid analgesic therapy, but patients, instead, become more sensitive to painful stimuli in many cases. Researchers interpreted that OIH is the consequence of opioid tolerance and probably the first sign of hyperalgesia. (Chang et al., 2007) The molecular mechanism is not yet well-characterized. Though a number of mechanisms have been assumed the general surmise is the neoplastic modifications in both peripheral and central nervous may lead to hyperalgesia. (Lee et al., 2011) The sign of OIH can be identified if there is a lack of pharmacological efficacious responses during management of chronic pain with opioids administrations. (Hayhurst & Durieux, 2016) This unwelcome OIH expression might be managed by administration of low-dose ketamine, methadone, a combination of morphine and dextromethorphan in 1:1 ratio, COX-2 inhibitors and also considering some other treatment strategies. (Lee et al., 2011)

Addiction, abuse, and dependence. Addiction is a chronic and relapsing brain disease where patients irresistibly seek drugs even though they know about the detrimental consequences. Anything which can produce the feelings of euphemism can lead to addiction and abuse, and this can be a drug, food, sex etc. (Savage et al., 2003) Most of the opioid agonists can produce a reward, and direct to addiction, abuse and creates drug

dependence. In general, dependence can be defined as the condition where the subject is not able to cut down or quit specific substance because of long-term use, despite trying harder and when it does happen with opioid drugs, called opioid dependence. (Jamison & Mao, 2015; Rosenblum et al., 2008) Both addiction and dependence happen over time to obtain a reward. Opioids addictive and dependent patients can do anything such as stealing money or drug, begging, robbing or intimidating anyone to administrate drugs.

Opioid withdrawal. Drug withdrawal is a bunch of symptoms which become obvious because of discontinuing or significant reduction all on a sudden of any substance with or without medical values which have been administrating. Usually, the drugs which can produce rewards may cause withdrawal disorders which might stay for a week or more after an abrupt massive interfering of the last dose. Withdrawal symptoms can occur after developing drug dependence. (Savage et al., 2003) Nausea, vomiting, muscle cramping, depression, anxiety, opiate cravings, agitation etc. are common withdrawal symptoms. (Hanks & Hoskin, 1987) This is a great challenge to overcome during opioids addiction and dependence treatment.

Neurobiological Processes in Drug Reward

Now the question is how a super opioid analgesic can create drug addiction, abuse, and dependence? To understand this neurobiological mechanism, we need to know the opioid receptor's location (*Figure 2*) and functions, the binding affinity of clinical opioid ligands to their receptors which have already been discussed. From the previous discussion, activating MOR, DOR and KOR is not only produces analgesic effects but also generates rewards feelings in the brain. Along with these, we need to the neuroanatomy of the reward pathway of the brain. The pleasure feelings come by the release of dopamine (DA) into

midbrain. (Fields & Margolis, 2015) Dopaminergic neurons are the principal source of DA which are numerous found in midbrain and 90% percent of these are located in ventral part of the mesencephalon. (Chinta & Andersen, 2005) There are four major dopamine pathways (*Figure 3*) by which DA can travel to different areas of the brain and body and convey messages like pleasure and reward, locomotion, thinking, cognition etc. (Adinoff, 2004; Fields & Margolis, 2015)

Mesolimbic Pathway: This pathway is involved in pleasure and reward functions. The dopamine enriched ventral tegmental area (VTA), initiates dopaminergic action potentials and sends the signals to another area of the brain, nucleus accumbens (NAc) (*Figure 3*). The release of dopamine in NAc primarily produces reward and pleasures. (Adinoff, 2004) Overstimulation of this pathway causes addiction, abuse, and dependence.

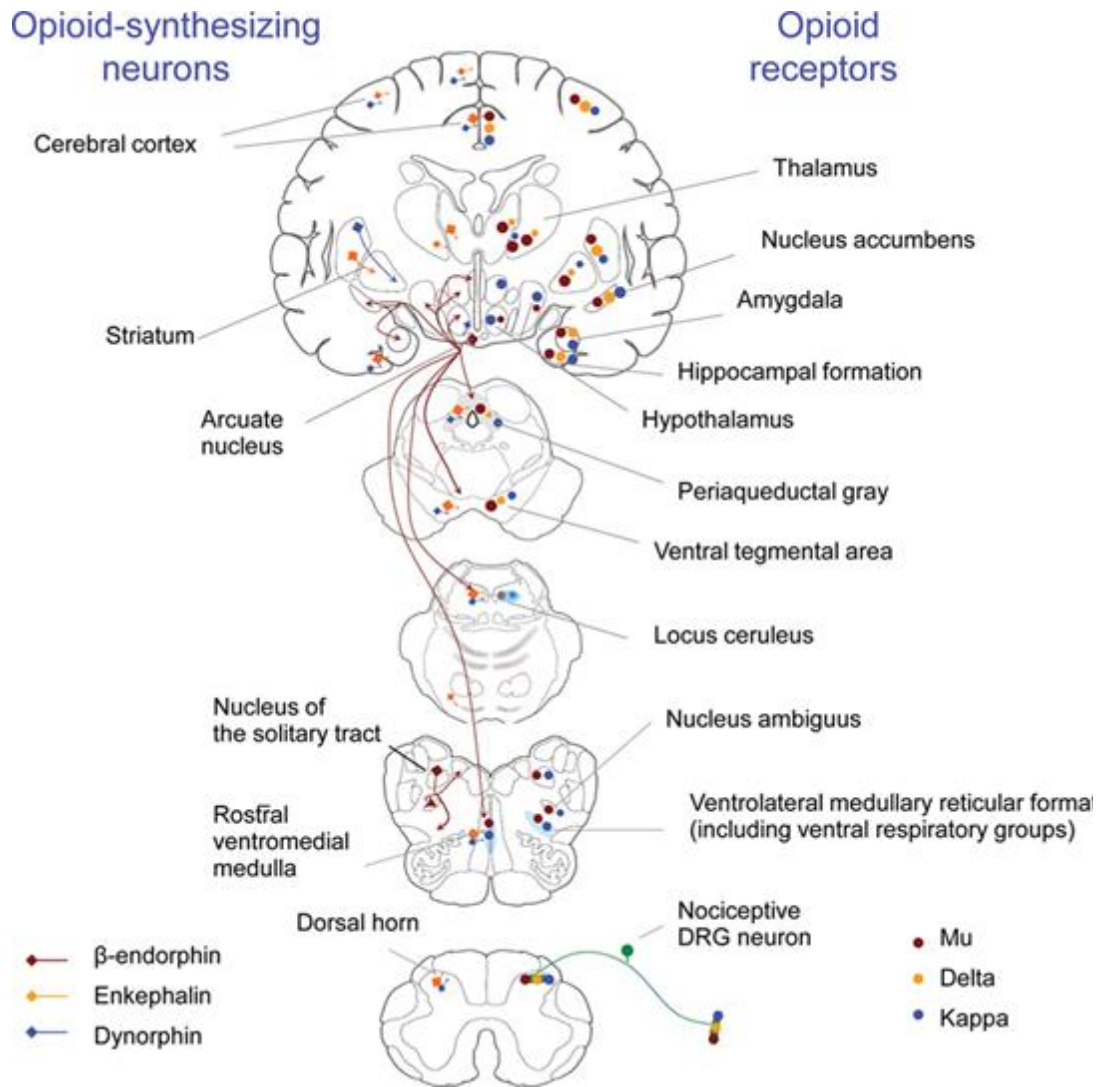


Figure 2. (Benarroch, 2012) The schematic illustration of the locations of opioid receptors and opioid-synthesizing neurons. The dopaminergic neurons (Figure 3) and opioid receptors shared some common locations of brain, specially, the VTA and NAc, that are reward producing area. Opioids agonists activate the mesolimbic dopamine pathway as their target receptors and dopamine neurons located same area. (Adinoff, 2004; Chinta & Andersen, 2005; Kosten & George, 2002)

Mesocortical pathway—dopamine is synthesized in VTA (*Figure 3*) transmits signals from the VTA to the prefrontal cortex (PFC) which is involved in memory, motivation and decision making (*Figure 3*). (Puig, Rose, Schmidt, & Freund, 2014) The dopamine

secretion in this area might be helpful in cognition behavior but it may also elevate the dopamine level in NAc by the mesolimbic pathway which will drive to addiction. (Yadav et al., 2014) The inappropriate function of this pathway may generate schizophrenia, ADHD (Attention Deficit Hyperactivity Disorder), and psychosis. (Puig et al., 2014)

Nigrostriatal pathway—dopamine sends signals from the substantia nigra to the basal ganglia which are associated with the movement (*Figure 3*). (Puig et al., 2014) Inadequate dopamine secretion in this pathway causes Parkinson's disease (PD).

The tuberoinfundibular pathway is the final dopamine pathway where dopamine is synthesized in the hypothalamus (*Figure 3*) and conveyed the signal in the pituitary which functions on regulating prolactin hormone secretion (*Figure 3*).

Opioid receptors are also numerous found in ventral area tegmental and nucleus accumbens (*Figure 2*), where dopaminergic receptors are located densely. So other than only to reduce nociception, opioid analgesics also produce euphoria by activating dopamine pathway and elevating dopamine in the midbrain. (Volkow & McLellan, 2016) When the mesolimbic pathway is activated, that primarily calls upon euphoric feelings and continuous euphemism leads to drug addiction, abuse, and drug dependence. (Adinoff, 2004; Chinta & Andersen, 2005) Therefore, the addiction and abuse of opioid drugs will be increased with the number of prescriptions for these medications.

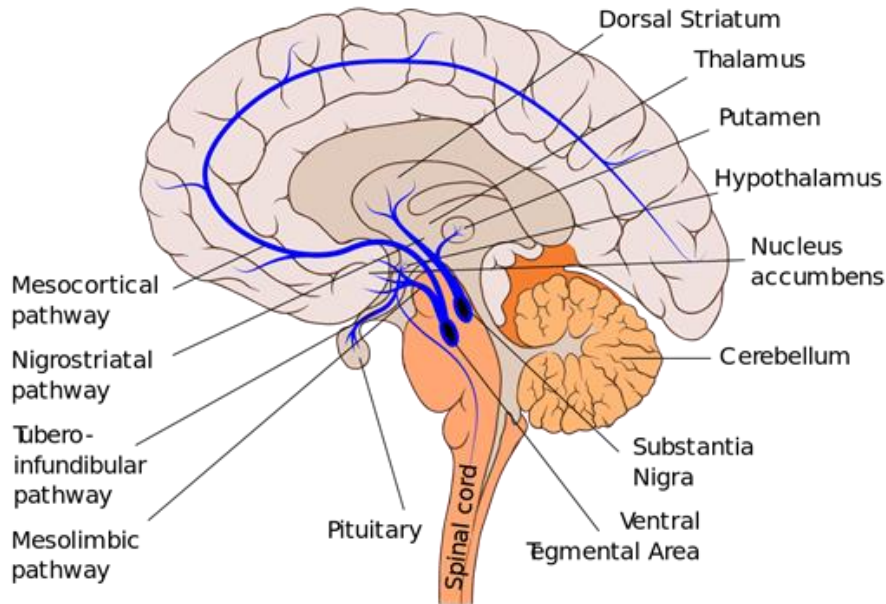


Figure 3. (Wikipedia, 2015) The dopaminergic pathways. Mesolimbic pathway responsible for reward & pleasure feeling which is the principal reason of drug addiction also; Mesocortical pathway control cognition and activity; Nigrostriatal pathway controls motor function and tuberoinfundibular pathway maintains prolactin secretion.(Benarroch, 2012; Wikipedia, 2015)

Addiction Statistics: Effects on Health and Economics

Opioid prescriptions have been increasing day by day, corresponding with an increase in opioid abuse. (Kuehn, 2007a) According to the very recent Annual Surveillance Report of Drug-Related Risks and Outcomes United States, in 2016, nearly 62 million people either filled or refilled opioids medications at least once, which was 19.1 percent of patients. (National Center for Injury Prevention and Control (NCIPC), 2017) The same report also shows: during 2015, opioid drug overdose caused 33,091 deaths and among them, 15,281 persons died from prescription opioid drug overdose.(National Center for Injury Prevention and Control (NCIPC), 2017)

The overall addiction scenario can be elucidated from results of the 2016 National Survey on Drug Use and Health. According to this report, approximately 28.6 million Americans aged 12 or more use illicit drug categories which include hallucinogens, inhalants, methamphetamine, and the misuse of prescription pain relievers, tranquilizers, stimulants, and sedatives. Among drug abusers, 3.3 million are pain relief prescription misusers. Approximately, in 2016, 11.5 million people (aged 12 or more) misused prescription pain relievers wherein 97.4 percent are opioid misusers also 1.9 million misused cocaine. Drug-induced death has been also increasing nearly steadily with time. In 2014, drug-induced deaths totaled 33,671; in 2015, the total increased 6.9% to 36, 2622 in 2015, and in 2016 it further increased by 5.7%. If we compare between 2006 and 2016, then the percent of death has been increased shockingly almost 46 percent. (Centers for Disease Control and Prevention, 2018)

Aside from the health issue, this is an extensive economic burden too. In 2007, the United States had to expend more than \$193 billion for illicit drug use, and surprisingly

only the prescription opioid abuse cost was approximately \$55.7 billion. (Center, 2011; Kuehn, 2007b) The overall scenario became worse in 2013 compare to 2007—wherein the economic burden was estimated to be \$78.5 billion, which included increased healthcare and criminal justice costs.(Florence, Zhou, Luo, & Xu, 2016)

Drug Addiction and Treatment

Detoxification of opioid drugs is the primary goal for the treatment of opioid addiction. The treatment is often possible either by behavioral therapy or pharmacotherapy or combination of both therapies.(Carroll & Onken, 2005)

Behavioral therapy. Upon based on the previous era, the success of behavioral therapy is significantly beneficial for treating drug addiction and abuse. There are different types of behavioral therapies: Cognitive behavioral therapy, contingency management, community reinforcement approach, motivational enhancement therapy, family behavioral therapy, and other behavioral approaches have been proved as worthwhile for managing different types of addictions.(Carroll & Onken, 2005; NIDA, 2018)

Cognitive-behavioral therapy. Relapse is a very common problem for any kind of treatment against. CBT which includes functional analysis and skill training can significantly help in the prevention of relapse. (Hendershot, Witkiewitz, George, & Marlatt, 2011) Patients can learn different techniques to discriminate correct and incorrect behaviors that help them approaching a problematic situation to abstain from drug abuse. (Steve, Wendy, & Vic, 2009) Individuals can develop adaptive strategies in unfavorable social conditions through specific skills such as eliciting knowledge about positive and negative consequences results of drug abuse, self-control about drug craving, avoiding possible vulnerable situation etc. (Carroll & Onken, 2005; NIDA, 2018; Steve et al., 2009)

In some addiction such as cocaine addiction, CBT can be more effective along with pharmacotherapy. (Carroll et al., 2004)

Contingency management (CM). This behavioral therapy is based on tangible rewards to encourage the abstinence from drug and the rewards can be either voucher-based reinforcement or prize incentive. (NIDA, 2018) If the drug test such as urine or breath test confirms drug-negativity, the patient will receive a monetary voucher (voucher-based reinforcement CM) or win cash instead of the voucher (prize incentives based CM), either way, allows the patient to exchange food, goods or any service to reinforce his/her drug-free life. (Budney, Higgins, Radonovich, & Novy, 2000) A considerable number of patients have abstained from opioids or cocaine patients through this CM service. (Petry et al., 2005; Prendergast, Podus, Finney, Greenwell, & Roll, 2006) Though initially, the community was concerned about increasing gambling because of prize incentives later it was confirmed that this service did not promote gambling. (Petry et al., 2006)

Community reinforcement approach (CRA). This behavioral therapy is 24-week therapy where patients also are rewarded with vouchers for treating cocaine and alcohol addicted people. (NIDA, 2018) Computer-based CRA is effective for opioids and/or cocaine-dependent patients. (Higgins et al., 2003) This version train adolescents about solving the problem, adjustment, communication skills and encouragement to participate in recreational activities. (Brooks, Ryder, Carise, & Kirby, 2010)

Other behavioral therapy with different strategies also become helpful with or without pharmacotherapy.

Pharmacotherapy.

Methadone. Methadone hydrochloride, a MOR agonist, is the first line of opioid pharmacotherapy which has been used since 1972 upon the approval by the Food and Drug Administration (FDA). (Institute of Medicine (US) Committee on Federal Regulation of Methadone Treatment; Rettig RA) Despite this is an opioid agonist but it does not generate pleasure feeling and the long-lasting pharmacological on the body, makes a drug of choice for treating opioid addiction and dependence. (Stotts, Dodrill, & Kosten, 2009) The elimination half-life of methadone is 24-36 hours which is optimal for a longer period of detoxification. The optimal dosing range for most of the patients is 60-150 mg/daily but the starting dose range is 20-30 mg/daily which can be gradually increased by 5-10 mg to catch the standard range. (Institute of Medicine (US) Committee on Federal Regulation of Methadone Treatment; Rettig RA; Stotts et al., 2009) The daily single dose of methadone can suppress the opioid withdrawal symptoms. Special precautions must be considered for the patients with chronic renal diseases and pregnant women and there is a very chance to grow methadone dependence on the fetus. (Institute of Medicine (US) Committee on Federal Regulation of Methadone Treatment; Rettig RA) Methadone pharmacotherapy is more effective with the combination of other behavioral therapy.

Buprenorphine. This synthetic opioid receptor partial agonist is another important medication for opioid dependence. Buprenorphine does not produce euphoria and can greatly reduce opioid withdrawal symptoms that can be safely prescribed by primary care physicians. (Kahan, Srivastava, Ordean, & Cirone, 2011) Two different sublingual tablet formulations—just buprenorphine and the combination of buprenorphine and naloxone are available which was first approved by FDA in 2002. (NIDA, 2018; Stotts et al., 2009) The

maximum recommended dose of buprenorphine is 24 or 36 mg. To reduce possible abuse liability, buprenorphine and naloxone ratio 4:1 often choose over only buprenorphine. (Mendelson et al., 1999) Commercially buprenorphine to naloxone 2:0.5 or 8:2 combination are available by the brand name Suboxone. (Kahan et al., 2011) One of a clinical trial showed that the ratio buprenorphine: naloxone, 8:2 and 32:8 mg is better than 2:0.5 mg in reducing heroin replacement therapy. (Mattick, Kimber, Breen, & Davoli, 2008) The treatment becomes more effective when behavioral therapy like extended weekly counseling is added with Suboxone administration. (Fiellin et al., 2006)

National Institute of Drug Abuse (NIDA) clearly explained, as methadone and buprenorphine both are opioid drugs, so the treatment with both of these is more likely as a substitution of one addictive drug to another but in less addictively.

Naltrexone. is an opioid antagonist which is synthetically obtained, can prevent opioid agonists to bind with their target receptors. (Stotts et al., 2009) It produces neither rewards nor considerable abuse or addiction which gradually detoxify opioids effects on the body. Once daily (must be taken with food) 50 mg tablet is a very common treatment though it can be extended to 100-150 mg in every two-three days—based on physician's judgment. (Krupitsky, Zvartau, & Woody, 2010; Stotts et al., 2009) Scientists have been trying to develop sustained release formulation, though some sustained release formulations were prepared, FDA denied the approvals. (Krupitsky et al., 2010; Stotts et al., 2009) It has been thought that the long-acting release of naltrexone can improve the treatment therapy.

All of these treatment options with methadone or buprenorphine detoxify slowly. The drug-like clonidine, an alpha 2 adrenergic agonist, often as a combination therapy with

opioid antagonist naltrexone, can undergo rapid opiate detoxifications. (Gowing, Ali, & White, 2000) Even though this treatment option sounds wonderful but the obtained data from different studies could not ensure the claimed efficacy for opioid addiction treatment. (Stotts et al., 2009)

Creating New Opioids

“Prevention is better than treatment”—this is an apothegm; A new opioid analgesic without or limited abuse liability and with a lower side-effect will be an ideal treatment option for managing pain. Unfortunately, the opioid choice is limited. It was always being demanding to create a new opioid analgesic with less adverse effect. The first single opioid compound, morphine, was isolated from the tarry poppy seed juice by pharmacist Friedrich Wilhelm Adam Serturner in 1805. (Krishnamurti & Rao, 2016) After 120 years long research of isolation, morphine’s structure was established in 1925 by Sir Robert Robinson and it took around 30 years to find out the laboratory total synthesis of morphine which was developed by Marshall D. Gates.(Bentley, 1987; Gates & Tschudi, 1956) Structural modification in some important positions by analyzing structure-activity relationships (SARs) in 4,5a-epoxymorphinan skeleton (*Figure 3*), in previous years, remarkably helped to create new opioids. (S. Majumdar et al., 2012; Pasternak & Pan, 2013)

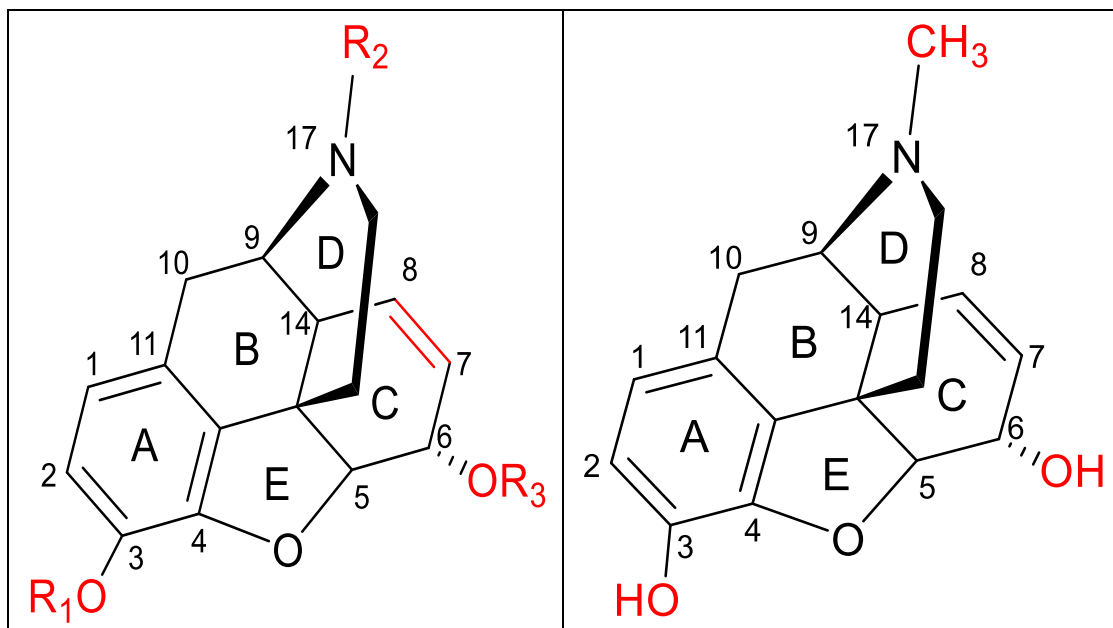
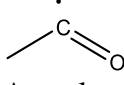
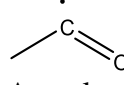


Figure 4. 4,5 α -epoxymorphinan template (left) and morphine (right). The SARs of morphinan compounds have been primarily created by altering substituents at the three R groups. (Pasternak & Pan, 2013)

Table 2

Examples of Some 4,5 α -epoxymorphinan Compound's SARs Modification at Different Carbon. (Pasternak & Pan, 2013)

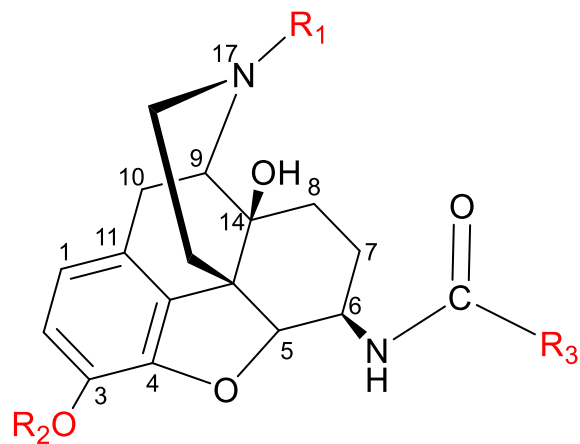
Compounds	R ₁	R ₂	R ₃
Morphine	H	CH ₃	H
Codeine	CH ₃	CH ₃	H
Morphine-6-sulfate	H	CH ₃	SO ₃
Morphine-6 β -glucuronide	H	CH ₃	Glucuronide
Heroin	 Acetyl	CH ₃	 Acetyl

Structural features. The 4,5 α -epoxymorphinan compounds (Table 2) have some common structural features: a benzene ring (A), cyclohexane rings (B and C) which are partially unsaturated, a piperidine ring (D) and with a dihydrofuran ring (E). A hydroxyl group especially at the C-3 position (*Figure 4*), play a vital role for narcotic analgesic effects, loss of free hydroxyl group significantly reduce the affinity of an opioid to MOR or it may produce some other effects such anti-tussive effect of codeine. (Pert, Pasternak, & Snyder, 1973) Change at C-6 (*Figure 4*) with the different group also can affect the overall pharmacological properties—such as when hydroxyl groups of morphine are replaced by acetyl at both C-3 and C-6 positions, it elevates the lipophilicity and enhances blood-brain barrier (BBB) permeability.(DeRuiter, Fall 2000; Pasternak & Pan, 2013) The reduction of the double bond at C7-C8 (*Figure 4*) yields dihydromorphine, further substitution at C-14 by a hydroxyl group and oxidation of hydroxyl group at C-6 produces oxymorphone with more activity. A basic, tertiary amine at position 17 (*Figure 4*) plays an important role too, substitution of oxymorphone's N-methyl group with an allyl group, makes an opioid antagonist, naloxone, and if replacement happens by an ethylcyclopropane it yields another opioid antagonist, naltrexone. A number of other modifications also have developed several different clinical opiates, including both antagonists and agonists (Table 2).

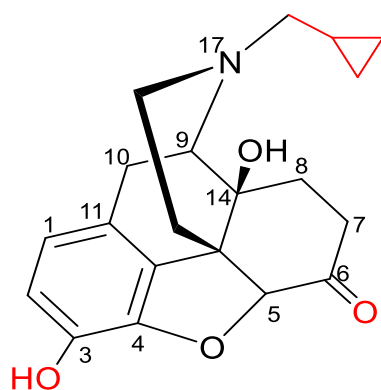
Chapter 2

A Novel Atypical Mu Opioid Receptor Agonist 3-Iodobenzoylnaltrexamide (IBNtxA)

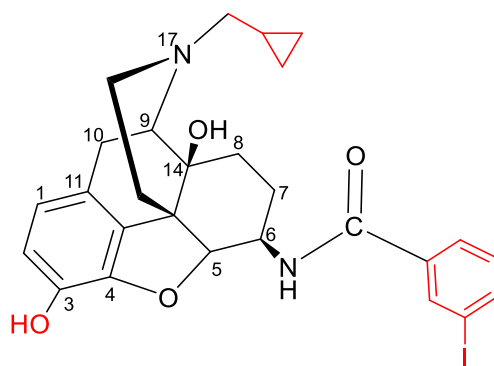
Scientists have been aspiring to generate novel opioid compounds with better analgesia but limited side-effects. Recently when a group of scientists from Memorial Sloan-Kettering Cancer Center, New York, had been synthesizing radiolabeled derivatives of the opiates, they found an atypical novel opiate, named- IBNtxA with wonderful pharmacological characteristics. (Susruta Majumdar, Burgman, et al., 2011; Susruta Majumdar, Grinnell, et al., 2011; S. Majumdar et al., 2012) This molecule was synthesized as a 6 β -naltrexamine derivative which is an analog of naltrexone. When they substituted R₃ position with 3-iodobenzene, R₁ with methylcyclopropane and R₂ with hydrogen, they found – IBNtxA (*Figure 5*). (S. Majumdar et al., 2012) Their investigation found—IBNtxA is potent than morphine when they tested analgesic properties by tail flick method, with lowered side-effects such as no respiratory effects, no physical dependence, and no place preference when they tested single dose. (Susruta Majumdar, Grinnell, et al., 2011) IBNtxA possibly signals through truncated MOR splice variants—exon 11-associated 6 transmembrane region splice variants (6TM/E11) but the role of 6TM/E11 is not well-established though it's been hypothesized that it can affect the analgesic signaling of some MOR agonists. (Lu et al., 2015; Susruta Majumdar, Grinnell, et al., 2011)



4,5-epoxymorphinan skeleton



Naltrexone



IBNtxA

Figure 5. SARs of 4,5-epoxymorphinan skeleton wherein the replacement of R_1 at N-17 position by methyl cyclopropane, R_2 at C-3 position by hydrogen and double bond with oxygen at C-6 position, produces opioid antagonist, naltrexone. Change in 4,5-epoxymorphinan skeleton at R_1 and R_2 same as naltrexone but replacement of R_3 by 3-iodobenzene creates an atypical mu opioid receptor agonist IBNtxA which is a derivative of 6 β -naltrexamine with higher analgesic effects but limited side effects than morphine and highly selective to 6TM/E11 MOR splice variant. (*S. Majumdar et al., 2012*)

Recent molecular dynamics simulations studies (led by Dr. Chun Wu) on morphine and IBNtxA with 7-transmembrane (7TM) and 6-transmembrane (6TM) of MOR splice variants, has confirmed that morphine is incapable to activate 6TM where IBNtxA can activate but the interaction between IBNtxA and 6TM/E11 and 7TM splice variants remains unclear.(Sader, Anant, & Wu, 2018) This analysis also confirmed that IBNtxA has stronger binding properties to 7TM than morphine.(Sader et al., 2018) The loss of exon 11-associated MOR splice variants in knock-out (KO) mice, caused loss of analgesia for IBNtxA but the analgesic effect of morphine was unchanged and when exon 1-associated splice variants, DOR and KOR were knocked out, morphine was unresponsive to those animals but IBNtxA expressed, analgesia—both event indicates that IBNtxA may response through 6TM/E11. (Susruta Majumdar, Grinnell, et al., 2011)

In this collaborative pilot projects we investigated: Analgesic properties of IBNtxA other than tail flick method, expanded abuse liability testing of IBNtxA using conditioned place preference (CPP), potential anti-addictive impact of IBNtxA by measuring its effects on morphine CPP, whether IBNtxA affects morphine-induced locomotion, the subjective effects of IBNtxA (MOR/KOR/DOR signaling?) by drug discrimination techniques and provide a foundation for future studies dissecting the effects of IBNtxA on other receptors and evaluating analogues of IBNtxA.

Materials and Methods

Animal. All experiments used male CD-1 mice or C57BL/6 mice obtained from Charles River Laboratories. All animals housed in the temperature- and humidity-controlled Cooper Medical School of Rowan University vivarium, a barrier facility, under a 12 h light/dark cycle (lights on at 0700, off at 1900). Mice were group housed in polycarbonate cages with *ad libitum* food and water and enrichment provided by paper Bio-Huts and/or nestlets. Mice arrived at the facility approximately 28 days of age and were allowed to equilibrate to the facility for a minimum of seven days before beginning testing.

CD-1 mice. CD-1 laboratory mice (*Figure 6*) are inexpensive and widely used in biomedical and pharmaceutical research. Most of the currently used mice are the progeny of nine Swiss mice, two male and seven female albino mice, which were imported to the USA in 1926 by Dr. Clara Lynch of the Rockefeller Institute for Medical Research, now named Rockefeller University.(Chia, Achilli, Festing, & Fisher, 2005) In 1948, new Ha/ICR mice were initiated from previously imported Rockefeller “Swiss” mice at the Institute for Cancer Research (ICR) in Philadelphia.(Chia et al., 2005)

Characteristics of ICR (CD-1®) Mice: CD-1 (*Figure 6*) mice are white in color; usually they are docile and these mice grow with time which became maximum after fifteen weeks though the growth rate and weight gain are higher in male than female.(River, 2018)

We started experiments when animals were around 35 days old, at that point we found the average weight of mice—approximately 34-36 gm. When we were working with CD-1 mice, in our observation, they were usually easy to handle but very first week they were outrageous, especially during drug administration. The reason behind their aggressive

behavior during the first few days—the time need for adaptation to human and drug administration.

C57BL/6 mice. This is a typical inbred strain, most widely genetically modified laboratory mice for biomedical, pharmaceutical, translational science or any animal study research due to their availability and robustness. This strain was first developed by C.C. Little in 1921 which was eventually handed over to Charles River in 1974 from NIH. (Chia et al., 2005; River, 2018; Sarna et al., 2000)

They are deep brown or almost black (*Figure 7*), highly sensitive to noise and odors; not docile like CD-1 mice and more likely to bite. They are barbering in nature, and dominant mice can remove hair and whisker of housemates. (Sarna et al., 2000) (Willott, Erway, Archer, & Harrison, 1995) Notwithstanding most of the different strains, these mice are highly susceptible to addiction, atherosclerosis and age-related hearing loss. (Willott et al., 1995) Like CD-1 mice, this strain also grows with time, reaching full weight after fifteen weeks; we started to weigh them after five weeks, and the average approximate weight was 18-22 g.



Figure 6:(Taconic Biosciences, 2018) Image of CD-1 outbred mouse, white in color and usually docile in behavior. These mice have been widely used in biomedical research. They are normal wild type mice, grow over time and gains maximum weight



Figure 4: (LABORATORY, 2018) Stock photo of C57 mouse, genetically designed animal. This strain is deep brown or almost black, noise and odors sensitive; are highly susceptible to addiction, atherosclerosis and age-related hearing loss. They are barbering in nature, prone to engage fighting with inmates, resulting hair removal and sometimes possible injuries. (River, 2018; Sarna, Dyck, & Whishaw, 2000; Zurita et al., 2011)

Drugs. IBNtxA was synthesized at Rowan University by the laboratory of Dr. Gustavo Moura-Letts, who developed a three-step synthesis starting from naltrexone purchased from Tocris. Morphine sulfate was purchased from Henry Schein. Cocaine HCl was purchased from Sigma. Naloxone was purchased from Tocris. The other substances that were used—naloxone, buprenorphine, methadone, U50,(488), TAN-67 (SB-205,607).

All drugs were delivered via intraperitoneal (i.p.) injection at a volume of 10 mL/kg. Drug dilutions were premixed to provide a given mg/kg dose when given an injection volume scaled to mouse body weight, measured prior to every test. For example, a 35 g mouse would receive a 1 mg/kg drug dose via the injection of a 0.35 mL volume of a 0.1 mg/mL drug solution. IBNtxA was delivered in a 10% DMSO vehicle, prepared via stepwise mixing with 1% 6M HCl, 10% dimethyl sulfoxide (DMSO), and 89% physiological saline. All other drugs were readily dissolved in physiological saline, or 10% DMSO vehicle. All the drugs were kept secure inside a locker with a regulated inventory procedure under the control Dr. Bradford Fischer, who holds controlled substances licenses from the State of New Jersey and the U.S. Drug Enforcement Agency.

Conditioned Place Preference (CPP)

Apparatus. The conditioned place preference (CPP) paradigm is a standard preclinical behavioral analyzing model which has been widely used for the investigations of abuse and addictions for illicit drugs, food, sex, etc. (Prus, James, & Rosecrans, 2009)

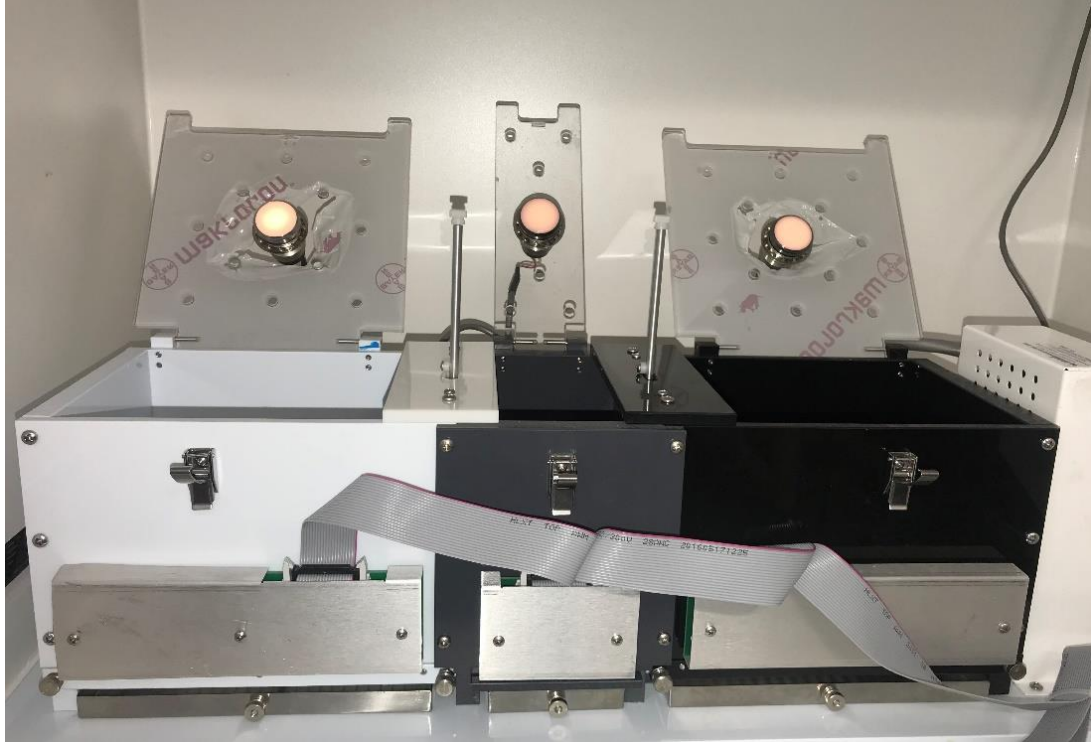


Figure 8: Photo of new CPP apparatus from our research lab, where in white chamber and black chamber for drug or vehicle-paired side and gray chamber in the middle is the neutral zone. The locomotor activity of animals is tracked by infrared and then signal is sent to MED-PC software to analyze and present on the monitor.

For IBNtxA-related CPP studies, we used modular CPP chambers from Stoelting for use with Any-Maze software. These chambers featured: with two rectangular shapes compartments which are connected through one small central compartment (*Figure 8*). Rectangular shaped compartments are either circular grid or square grid flooring with the similar marked wall. For *M. vaccae*-related studies, we used dedicated CPP chambers from Med-Associates for use with MedPC software. These chambers featured: three compartments in a rectangular box-shaped chamber, characterized by the white and black wall for two adjacent chambers of the center chamber which has a gray color wall (*Figure 8*). The center compartment doesn't have specific features, neither paired with drug nor with the vehicle, and two gates between two adjacent compartments connected with this compartment which allows animals to move freely.

General procedure. Initial preference: Before starting the training, we took consideration whether animals have any initial preference to any chamber. For instance, we named two different compartments, suppose, circular grid compartment and square grid compartment. Prior to conditioning, we calculated the ratio of time spent in an individual compartment. Usually, in the unbiased experimental procedure, the drug-paired, and vehicle paired compartments are assigned randomly regardless of initial preference score but in a biased CPP study, the compartment which is least preferred by subject is paired with the drug for that individual. (Huston, Silva, Topic, & Müller, 2013)

Drug conditioning: during conditioning (*Figure 10*), which also can be defined as training or acquisition, animals are repeatedly and alternately exposed to either the investigative drug while confined to one compartment or vehicle while confined to the other compartment. (Huston et al., 2013)

After assigned drug side and vehicle side for the individual, the mouse was confined to drug-paired compartment after injecting with drug and, in alternate sessions, the same animal was confined to vehicle paired compartment after getting vehicle injections (*Figure 10*). Before drug/vehicle administration individual animal's health and behavioral parameters—food intakes, percent of weight gained or lost, any injury due to fighting with mates, any infection for any possible reason, quality of stool, hyper or hypo activity—were noted and scored. Any animal which with considerable behavioral or health issue like sickness was separated and excluded from further experiments. The animals with good physiological conditions were then injected and placed inside pre-cleaned and properly set the CPP chamber. For the development of a place preference, animals were trained as long as 10 days acquisition period (*Figure 9*). Every time before the animal was placed inside CPP chamber, all apparatus was cleaned with 70% IPA and the beds were washed with water then cleaned by 70% IPA to eradicate microorganisms and get rid of from any possible previous odors.

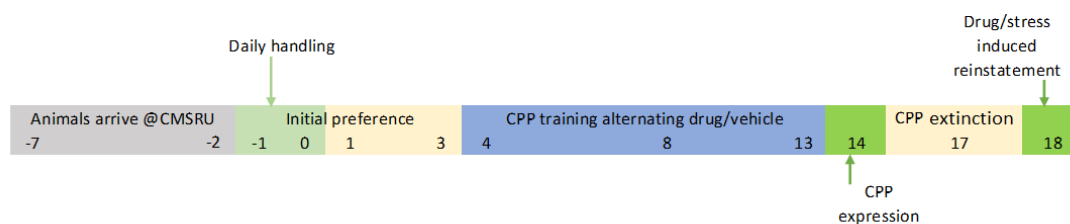


Figure 95: The schematic illustration of CPP test. The animals arrive in vivarium of Cooper Medical School of Rowan University (CMSRU) and have been kept for five days. After two days of daily handling to make them comfortable with researchers, they are being placed in CPP chambers for initial preference for two sessions then ten days (ten sessions) CPP training. After successfully completing training they have been tested for CPP expression and finally stress/drug-induced reinstatement test followed by CPP extinction.

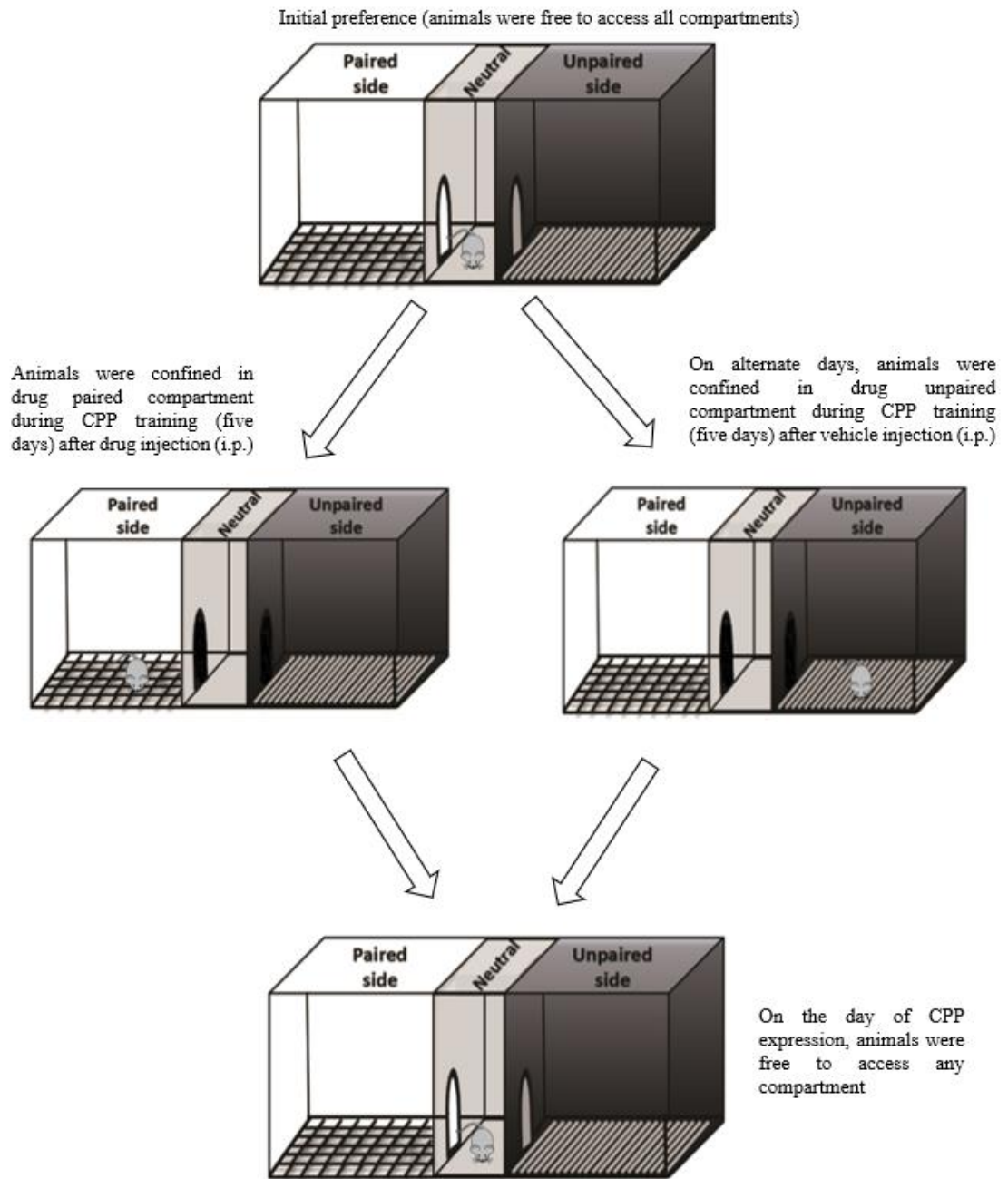


Figure 10. The general schematic presentation of CPP procedure. This figure was adapted from (Fernandes & Fulton, 2016).

The trained mice were tested to analyze place preference score through CPP expression trial, during this phase subjects did not receive any injection, and they were free to move any compartment of entire apparatus (*Figure 10*).

After completing CPP acquisition and expression (*Figure 9*), the conditioned mice were repeatedly exposed to all CPP compartments freely in absence of drug or vehicle. Three days of continuous trials led animals to loss of place preference—known as CPP extinction(*Figure 10*). (Prus et al., 2009)

CPP reinstatement (*Figure 9 &10*), followed by extinction, can be induced by either re-exposure to the drug which is also defined as drug-primed reinstatement or stress. Our pilot project had analyzed drug reinstatement study through both ways which were used as a model of relapse.

Two methods are widely followed for behavioral studies to investigate stress-induced reinstatement: forced swim and foot-shock; our studies were designed for forced swim stress-induced reinstatement. (Can et al., 2012; Yavin Shaham, Uri Shalev, Lin Lu, Harriet de Wit, & Jane Stewart, 2003) Mice were placed in an inescapable cylindrical tank which was 30 cm height x 20 cm diameters and constructed of transparent Plexiglas. The water level was marked on the tank which was more than 15 cm but less than 20 cm, and the water temperature was maintained 25 to 28 degrees Celsius. One mouse was kept for force swim in one cylinder for 5-6 minutes and then was dried and placed in CPP. The stresses mice were used to test the anti-stress, anti-anxiety as well as anti-addiction effects of drugs.

Statistical analysis. A preference score is calculated from the difference between the time spent in the drug-paired compartment before CPP training and the time spent in the drug-paired compartment on test day after CPP training—during CPP expression.

$$\text{Preference Score} = \text{time}_{\text{drug side}} - \text{time}_{\text{vehicle side}}$$

Drug Discrimination

Animal and drug. CD-1 male mice (*Figure 6*) were used for drug discrimination studies. Mice were food restricted for 6-12 hours prior to experiments but they have adequate access to water and air. Two group animal were used: one group was trained with morphine 3mg/kg and DMSO vehicle (1% 6M HCl, 10% DMSO and 89% saline), well-trained animals were tested with novel drug (1-3 mg/kg IBNtxA and 0.1, 1, 3 and 10mg/kg morphine and another group received training with 3mg/kg IBNtxA and vehicle, and trained animals were tested with morphine (1-30 mg/kg), buprenorphine (0.1-3 mg/kg) a non-selective MOR/DOR/KOR agonist and fentanyl (0.01-0.3 mg/kg) a MOR-1 agonist, U50,488 a KOR-selective agonist, (1-20 mg/kg), a DOR-selective agonist, TAN-67 (SB-205,607) morphine (1- 30 mg/kg), a MOR-1 agonist.

Apparatus. Drug discrimination study was designed in the murine model, to understand whether the subjective effects of IBNtxA were similar to morphine. The DD apparatus has consisted of eight small boxes (*Figure 11*) which were designed by cutting edge technology. Each small box made of acrylic transparent glass which was covered by a spacious larger wooden box. All boxes were connected via different cables with computers where MED-PC software converted the animals' behavioral activity to data and showed on the monitor.

Each box had two nose poke holes: one for drug and another one for vehicle side (*Figure 11*). When animals nose poked on either side, an infrared beam was broken and a

signal was sent to the operating MED-PC software. During the experimental session, boxes were enclosed to isolate animals from other outside environmental factors, but during confinement, they received adequate air and light. A liquid dipper was located between the two nose poke holes, connected via tubing to a syringe (*Figure 11*) that discharged vanilla Ensure, a palatable food reward, for 3 seconds (delivering an approximate 0.1 mL volume) as a reward when animals earned a programmed reward. To earn the reward, animals were required to complete a specific pattern of correct responses: the required number of correct responses to earn a reward is known as the fixed ratio (FR). An FR10 training paradigm, for example, requires that the animal complete 10 correct nose pokes in a row to earn a reward. MED-PC software regulated the total system.

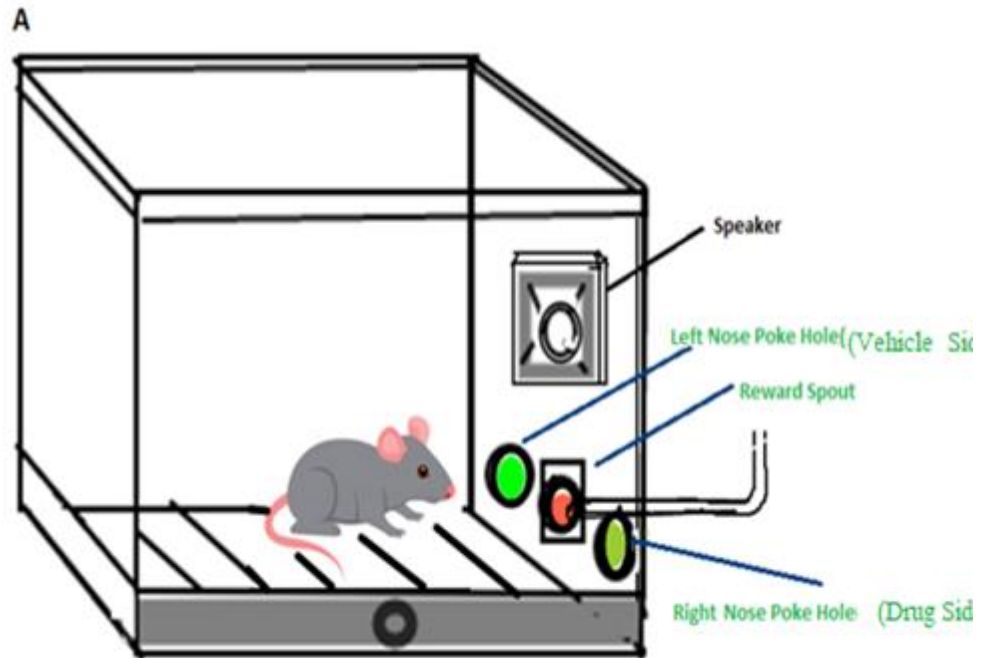


Figure 61. Schematic illustration of drug discrimination apparatus. The left hole is vehicle-paired; if animals knock this hole on vehicle-training session is considered as correct response and the right hole is drug-paired; on drug-training session if animals knock this hole, is considered as correct response. For every ten correct nose pokes, animals receive one single reward which is three seconds Ensure Plus syrup discharge through reward spout. During substitution test day any nose pokes to either side are considered for reward. The speaker on top of image is a sound generator which produces a tone during reward delivery.

Procedure. Animals were food restricted up to 24h before training or testing. During food restriction, animals have *ad lib* access to water. The weight of food-restricted animals was measured, and other visible health parameters were evaluated, including stool, appearances, and physical hyper/hypo-activities. After setting everything based on protocol, animals were injected and placed inside the boxes for training or test.

In our FR schedule, the subject must complete a set number of correct responses during the training period to obtain one reward, and our research considered maximum ten correct responses as FR to get a single reward. Fixed ratio ten means if animals knock ten times to correct side they will get one reward. For elucidation, when a mouse received training drug, the correct responses were considered if that animal hit on drug side and there was no reward for the incorrect response that means vehicle side response. The procedure was almost the same as the training session for substitute test except animals were free to choose any side for their rewards.

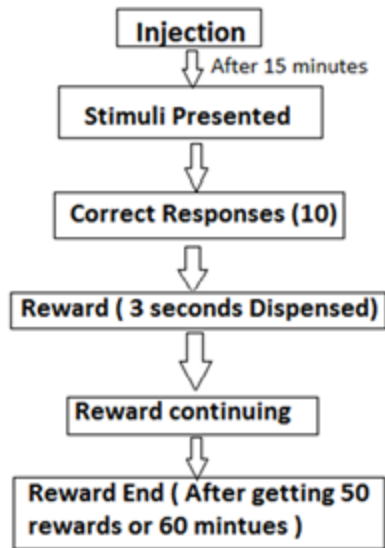


Figure 72. Outline of drug discrimination training. From the top of this image, after getting injection (i.p.) animals are placed inside DD box, and after 15 mins stimuli appears. Subject receives reward of three seconds liquid food dispense through reward spout for every ten correct responses. The training session becomes automatically end, either getting 50 rewards or after 60 mins or both.

Total procedure can be showed as this way (*Figure 12*): Injection (i.p.) > stimuli present (after 15 minutes) > response to correct side (FR=10) > Reward (3 sec chocolate syrup discharge) > end of single training session (Either after 50 reward and/or 60 minutes) . (Solinas, Panlilio, Justinova, Yasar, & Goldberg, 2006) At the end of each day experiment, all apparatus was cleaned with animal sanitary napkins, 70% IPA and water.

Two groups animals—each group contained eight CD-1 mice has been trained. One group was trained with vehicle (1% 6M HCl, 10% DMSO and 89% NaCl) and morphine 3 mg/kg. To train mice properly and unbiasedly, the pattern of training was always being changed in each week for overcoming any possible effect of training schedule pattern which might affect discrimination study.

The animals which could not reach the standard training with minimal 80% initial correct, 80% total correct response and 80% reward were excluded beforehand. Furthermore, every animal was kept in close observation for any health issue such as weight loss, stool condition, any possible injuries etc. Sick animals were separated from other mates and treated with required medicines. The animals with better health were trained and tested only.

Statistical analysis. Following equations were used for analysis:

For training.

% of total correct responses

$$= \left(\frac{\text{Total correct responses}}{\text{Total correct responses} + \text{Total incorrect responses}} \right) \times 100$$

% of initial correct responses

$$= \left(\frac{\text{Initial correct responses}}{\text{Initial correct responses} + \text{Initial incorrect responses}} \right) \times 100$$

For the substitution test.

% of total drug side responses

$$= \left(\frac{\text{Total drug side responses}}{\text{Total drug side responses} + \text{Total vehicle side responses}} \right) \times 100$$

% of initial drug side response

$$= \left(\frac{\text{Initial drug side responses}}{\text{Initial drug side responses} + \text{Initial vehicle side responses}} \right) \times 100$$

For the rate of response. Other than only considering percent of the response to drug/vehicle side, the response rate is also a crucial factor for drug discrimination study. A consistent and proper response rate confirmed the appropriate training dose and the possible range of dose which might be tested for a drug. Furthermore, the rate of response helped to analyze some certain behavioral study of animals such as sedation, hyperactivity or hypoactivity etc. for DD study. ("Frontiers in Neuroscience," 2009)

The simple equation is as follows:

$$\text{Response rate} = \frac{\text{Total number of responses}}{\text{Total time}}$$

Analgesic Test: Hot Plate Technique

Animal and drug. Male CD-1 mice, 40-50 days old, obtained from Charles River, had free access to food and water, exposed to a light-dark cycle of 12 h were used for this test.

Hot plate test was used to evaluate the analgesia of IBNtxA and then to compare with morphine. Novel drug IBNtxA 1 mg/kg and 3 mg/kg and morphine 10mg/kg were administered by intraperitoneal (i.p.) injection. Three groups of animals, each containing eight animals, were assigned to receive 1 mg/kg IBNtxA, 3 mg/kg IBNtxA or 10 mg/kg morphine.

Apparatus. The hot plate analgesia meter (Columbus Instruments, OH, U.S.A.) for small laboratory animals were used for this analgesic test. The hot plate could continuously provide 55°C temperature on an aluminum surface, with a digital built-in thermometer to maintain surface temperature to 0.1 °C precision and a timer with a 0.1 sec precision. The square shaped surface plate was enclosed by a clear acrylic cage to confine animals during testing. Pushes on start/stop button related to the timer, which displayed the time on the screen which was recorded manually.

Procedure. The hot plate was set at 56 °C to observe the effects of drugs on animals. Certain behavioral changes, paw licking, flutter, and jumping, were considered as an animal's pain feeling. (Rezaee-Asl, Sabour, Nikoui, Ostadhadi, & Bakhtiarian, 2014) Latency time after placing mice on the metallic hot plate provided the threshold level of animals. Prior to injecting the drug, each mouse was weighed and tested for two baseline studies where the animal was not injected with any drug or vehicle. After baseline studies, testing drug was administered and animals were placed on hot plate in 15, 30, 45, 60,

and/or 75/90 minutes time intervals to collect the latency time. If any behavioral change like paw licking, flutter or jumping had been observed, the mouse was immediately removed from hot plate and latency time was recorded. Animals were removed from hot plate after 20 seconds even though there was no considerable behavioral change to avoid tissue damage and this specific time is known as maximum latency time. Any animal which showed more latency time more than 20 seconds was excluded from further investigation. (Menéndez, Lastra, Hidalgo, & Baamonde, 2002; Rezaee-Asl et al., 2014)

Statistical Analysis. The anti-nociceptive effect for each dose was calculated as the % of the Maximal Possible Effect (% MPE) using the following formula:

$$\% MPE = \left(\frac{\text{Latency time after drug administration} - \text{Mean latency time of baseline studies}}{\{\text{Maximal latency time (20)} - \text{Mean latency time of baseline studies}\}} \right) \times 100$$

Open-Field Locomotion Test

Animal and drug. Male C57 mice were used and IBNtxA 3 mg/kg and and morphine 10mg/kg were administrated by intraperitoneal (i.p.) injection. There are two types of vehicles had been used: 1% 6M HCl, 10% dimethyl sulfoxide (DMSO), and 89% physiological saline (DMSO vehicle) and only physiological saline (0.9 % NaCl). Three groups of animals, each containing eight animals, were assigned to receive 1 mg/kg IBNtxA, 3 mg/kg IBNtxA or 10 mg/kg morphine.

Apparatus. The open-field locomotor test was determined by using 40*40*35 cm Plexiglas® open-field (*Figure 13*) and a camera mounted overhead, recorded and tracked locomotion of animals which was connected to the Any-maze behavioral analysis software of a computer. The field was divided by two regions: center region by 20*20 cm and rest of area for outer regions.

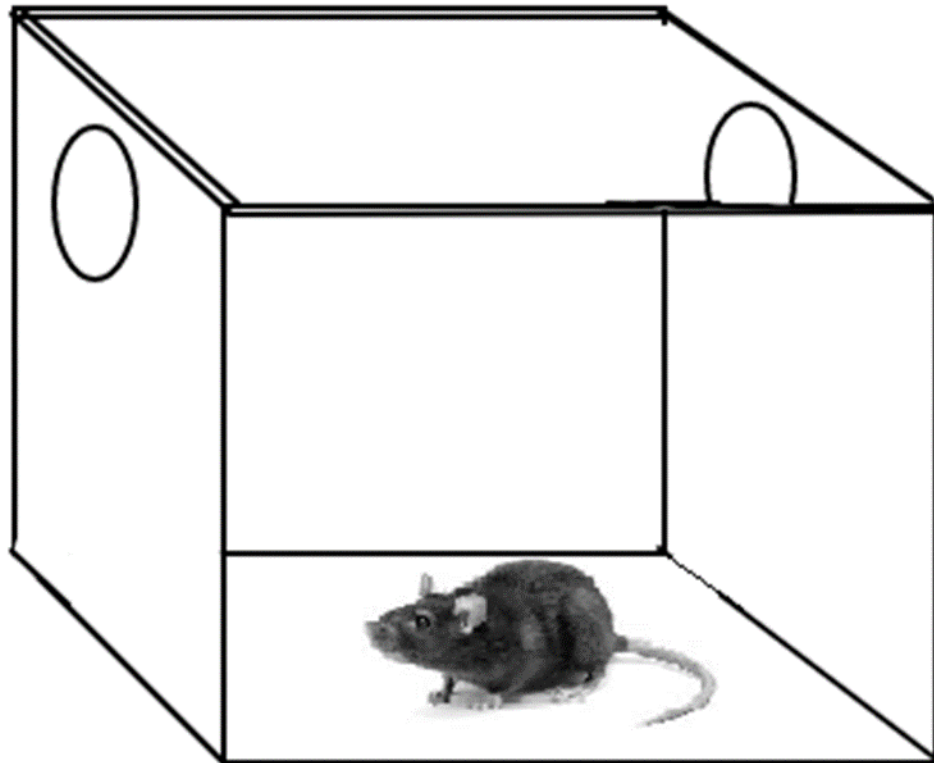


Figure 83. Schematic presentation of open-field box which is 40×40×35 cm Plexiglas® with a camera on the top of box is connected through cable with computer operated Any-maze software which tracks and analyzes animal's locomotor activity. The total open-field is separated into two zones—center zone (20×20 cm) and outer zone.

Procedure. The procedure was simple—every animal was pre-weighed before treated with vehicle or drug then was placed inside the confined open-field for 40 mins to investigate the locomotor activity.

To understand the effect of novel drug (IBNtxA) on morphine-induced locomotion, at first animals were injected with either IBNtxA or DMSO vehicle (1% 6 M HCl, 10% DMSO, 89% saline), were kept in home cages for 15 minutes then they were injected (i.p.) with morphine and were placed on field for 40 mins. The wall of boxes and beds were cleaned and dried every time for each animal's testing with 70% IPA to avoid possible microorganism contamination as well as previous any kind of smell.

Statistical Analysis. The total distance, time in the center zone, and time in outer zones were collected for further behavioral analysis. Total distance traveled by animals was statically analyzed using GraphPad Prism 6.0 statistical analysis software.

Results

Hot plate Analgesic Test: IBNtxA is more potent than morphine. According to the description above, the three group (n=8) of C-57 mice were injected intraperitoneally with two different strength of IBNtxA—1 mg/kg and morphine 10 mg/kg. When we compared the percent maximal possible effect (MPE) in the hotplate analgesic test of novel IBNtxA with classical MOR agonist analgesic morphine, we found that 3 mg/kg IBNtxA was more potent than 10 mg/kg morphine but 1 mg/kg IBNtxA did not show any considerable efficacy (*Figure 14*). Both IBNtxA 3mg/kg and morphine 10 mg/kg exhibited their maximum pharmacological action after 30 mins, wherein novel drug showed more than threefold more analgesia than control drug. In term of duration of action, the therapeutic curve indicated that morphine had a longer analgesic effect than IBNtxA.

Morphine took more than 90 mins to be reduced in zero percent MPE but IBNtxA took 60 mins. The possible reasons behind the difference between the duration of action between two drugs—half-life, protein bindings, and metabolism.

Conditioned Place Preference: IBNtxA does not induce a place preference

Using the method described previously, nine groups of C57 mice (n=6-15 for each group) were tested for morphine- or IBNtxA mediated place preference. Animals were administered (i.p.) morphine, IBNtxA, or vehicle in a two-compartment CPP chamber. One group received normal biological saline, four groups received different doses (0.1, 0.3, 1 and 3 mg/kg) of IBNtxA, another four groups of mice received four different doses (1, 3, 10, and 20 mg/kg) of morphine. Animals were assigned for drugs or saline administration in an unbiased method. According to our research plan, we compared the preference score of among control (saline), well-known MOR analgesic (morphine) and the novel atypical MOR agonist (IBNtxA). IBNtxA did not show any statistically significant preference score compared to morphine (*Figure 15*). The morphine-induced place preference score of was initially increasing with the increase of dose which decreased later in higher dose: there was no preference for 1 mg/kg, little preference score but nonsignificant for 3 mg/kg and high preference score for 10 mg/kg which was more than 20 mg/kg morphine dose. The comparison of preference score between 3 mg/kg IBNtxA and 10 mg/kg morphine (analgesic test showed both are effective and equivalent) exhibited significant CPP score difference wherein IBNtxA did not exhibit preference but morphine had highest preference score (*Figure 15*).

Conditioned Place Preference: IBNtxA reduces morphine-induced CPP. To investigate the effect of IBNtxA on morphine-induced CPP, three groups (n=10-11 each) of animals were used. Where all animals received morphine 10 mg/kg, but the pre-treatment substances were different for the three groups. The pretreatment time was 15 mins prior to inject (i.p.) morphine wherein first group was pre-injected with saline, the second group was pretreated with IBNtxA 1 mg/kg and the third group were pre-injected with IBNtxA 3 mg/kg. The result showed that mice those were pretreated with saline and IBNtxA, showed a preference for the drug side which indicated, IBNtxA 1mg/kg did not reduce the preference score (*Figure 16*). Animals which were pre-treated with IBNtxA 3 mg/kg, did not show any significant preference score which indicated that IBNtxA 3mg/kg significantly reduced the morphine-induced conditioned place preference score (*Figure 16*).

Open-field locomotor test: IBNtxA attenuated morphine-induced hyperlocomotion

Four groups (n= 6-11 per group) of C57 mice were used for this investigation. There were two stages of injection: pre-injection and post-injection—pre-injection were administrated 15 mins prior to get post-injection. Four groups of animals were assigned drug and vehicle according to the following ways—Group-A: pre-injection with IBNtxA 3 mg/kg and post-injection with morphine 10 mg/kg, Group-B: pre-injection with DMSO vehicle and post-injection with morphine 10 mg/kg, Group-C: pre-injection with IBNtxA and post-injection with saline and Group-D: pre-treatment with DMSO vehicle and post-injection with saline. The locomotor activity of Group-A, C and D were lower, but Group-B showed higher locomotor activity which clearly exhibited the morphine-induced hyperlocomotion (*Figure*

17). The animals which were pre-treated with IBNtxA 3 mg/kg prior to receiving morphine 10 mg/kg, showed normal locomotor activity like Group-C and D wherein animals were treated with vehicle, saline or IBNtxA (*Figure 17*). Further data analysis among all groups indicated that IBNtxA reduced the hyperlocomotion activity induced by morphine 10 mg/kg, moreover, IBNtxA did not have hyperlocomotion (*Figure 17*).

Drug Discrimination Study: IBNtxA partially substitutes for morphine

According to the method mentioned earlier, animals (n=8) were trained with morphine 3 mg/kg were tested for drug substitution of IBNtxA. It took almost 60 days to train animals well and then in each week one single dose of the even for the same drug was tested. When animals were well-trained (more than 80% correct response), they were tested with DMSO vehicle, morphine (0.3, 1, 3, 10 mg/kg) and IBNtxA (effective analgesic dose 3 mg/kg). The response to drug side was increased with the increase of a dose of morphine (*Figure 18*). The animals when pre-injected with naloxone 1 mg/kg before injecting morphine 3 mg/kg and asked to choose drug or vehicle side and they did not respond to the morphine side (*Figure 18*) which indicated that naloxone blocked the morphine to bind with target receptors. Finally, animals were tested for discriminating of IBNtxA with morphine and the result showed IBNtxA partially substituted morphine (*Figure 18*).

The response rate is critical to evaluate alongside the substitution results. Drug doses that substantially suppress behavioral responding can complicate interpretation of substitution results. The average response rates during the last four days of training with both morphine and vehicle were very similar, but slower than the average response rates of vehicle and low doses of morphine during substitution tests. During the test with

morphine, the rate of response curve was fallen downward when the dose was increased from 1 mg/kg to 10 mg kg but lower dose morphine caused high response rate (*Figure 19*).

Discussion

IBNtxA has a powerful analgesic effect, comparable to morphine, which is very crucial for alleviating moderate to severe pain. The patients with severe pain—i.e., those with cancer or major surgery—need potent analgesic. IBNtxA was effective at a lower dose compared to morphine. Our hot plate study has confirmed that IBNtxA induced analgesia comparable to morphine at a dose that did not produce conditioned place preference (*Figure 14*). A drug with potent analgesic effect with no or minimum addiction is very important to reduce addiction, abuse, and dependence. Place preference score, in a rodent model, has been widely used to correlate with the addiction ability of a drug. Our investigation expanded the abuse liability studies of IBNtxA by using preference score induced different doses which were compared with different doses of morphine-induced place preference scores (*Figure 15*). Since IBNtxA did not produce any considerable place preference, it might not have an addiction or abuse liability.

There are very few drugs such as buprenorphine, naltrexone and methadone are available for opioid replacement therapy or adjuvant therapy for opioid addiction. Our research has confirmed that IBNtxA significantly reduces the morphine-induced CPP (*Figure 15*) which is correlated with the anti-addiction potentiality of IBNtxA. Moreover, we found that it also suppressed the morphine-induced hyperlocomotion (*Figure 17*) that is also some sort of related to drug seeking behavior. Since IBNtxA suppresses both CPP and hyperlocomotion which are induced by morphine, so it might have potent anti-

addiction properties which could be used as an opioid replacement therapy for opioid addiction pharmacotherapy.

As a novel atypical MOR agonist, we have been investigating how IBNtxA signaling through, what are the

subjective effects of this drug. The drug discrimination study which we have been conducting to find out these questions. The part of this study has already been completed, showed that IBNtxA partially substituted morphine (*Figure 18*). The previous studies conjectured that it might be signaling through truncated E11/6TM splice variant of mu opioid receptor, but morphine does not signal through this splice variant, rather, it signals by full MOR. So, to know more about IBNtxA's subjective effects, one group of animals is currently being trained to discriminate 3 mg/kg IBNtxA from DMSO vehicle. Once fully trained, they will be tested for drug substitution with morphine (1-30 mg/kg), buprenorphine (0.1-3 mg/kg), fentanyl (0.01-0.3 mg/kg), U50,488 (1-20 mg/kg), and TAN-67 (SB-205,607, 1-20 mg/kg). All the substitute results will soon confirm the subjective effects of IBNtxA.

Conclusions

The opioid epidemic is now a widely known term for America as well as other parts of the world. America has been fighting against this crisis for a longer time. Our collaborative research has confirmed that IBNtxA does not have possible addiction liability through our extended CPP test. Moreover, we also confirmed the possible anti-addiction ability of this drug along reconfirmed the stronger analgesic effect of IBNtxA. We are very close to finding out the subjective effect of this molecule which will be providing more obvious information for understanding IBNtxA signaling mechanism. A new clinical

opioid analgesic with higher potentiality but lower side-effect could be a groundbreaking medicine to fight against opioid addiction.

Hot plate analgesia

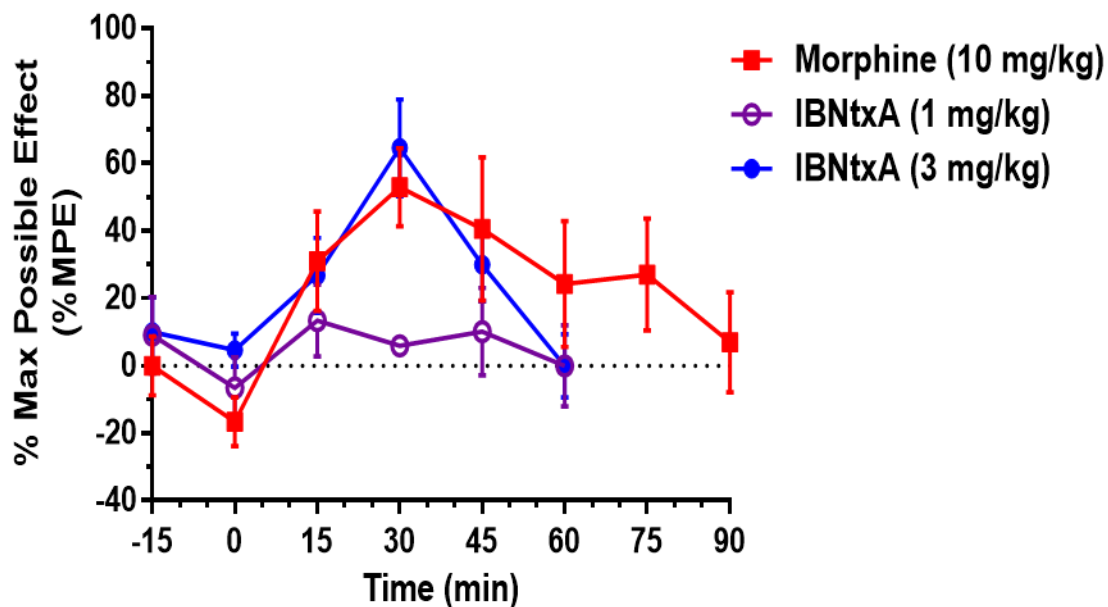


Figure 94. IBNtxA hot plate analgesia in CD-1 mice. Three groups of mice (n = 8 each) received 1 mg/kg IBNtxA, 3 mg/kg IBNtxA, or 10 mg/kg morphine i.p., and were tested independently on a 56° C hot plate. 30 min after injection (time = 0 min), 3 mg/kg IBNtxA and 10 mg/kg morphine showed peak analgesic effects. 1 mg/kg IBNtxA did not show an analgesic effect. From the dose-response above, it can be inferred that 3 mg/kg IBNtxA is comparable in analgesic potency to 10 mg/kg morphine. Results were evaluated as

$$\% MPE = \left(\frac{\text{Latency time after drug administration} - \text{Mean latency time of baseline studies}}{\{\text{Maximal latency time (20)} - \text{Mean latency time of baseline studies}\}} \right) \times 100.$$

All data are presented as means \pm SEM.

IBNtxA & Morphine place preference

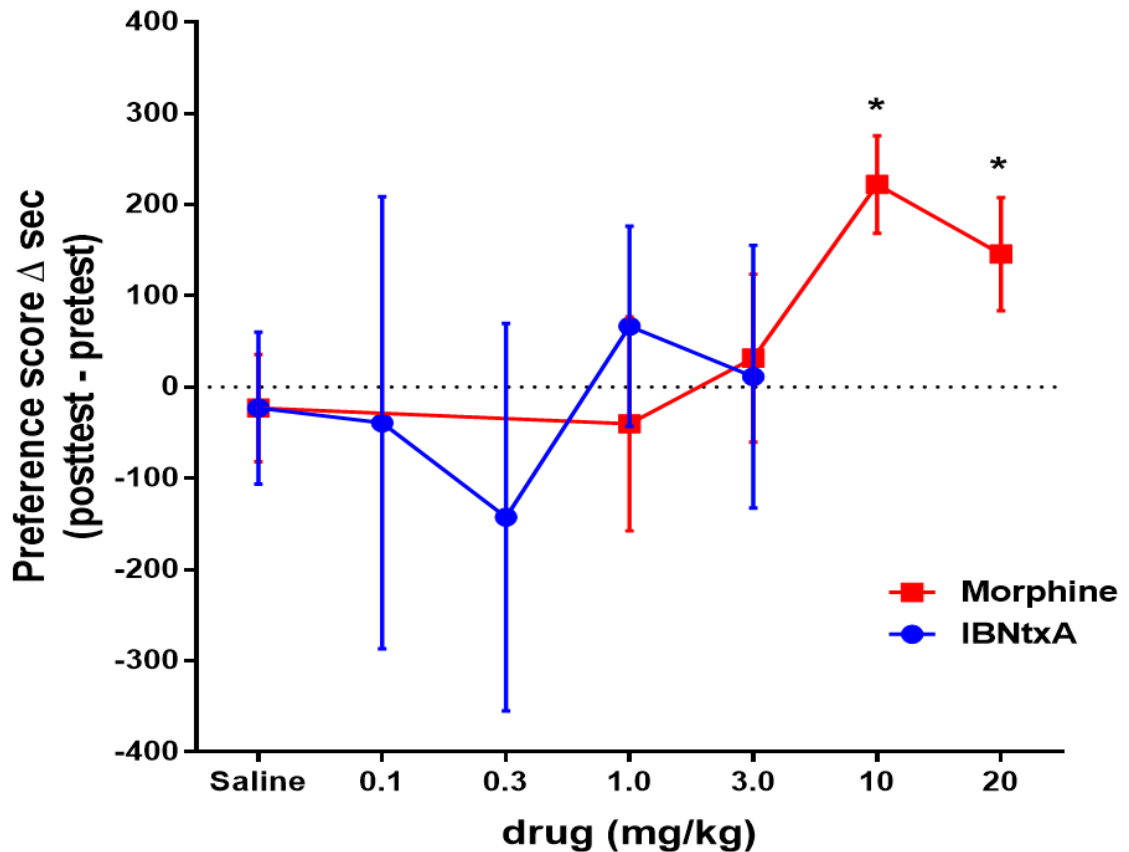


Figure 105. CPP scores comparison among saline, morphine, and IBNtxA. One group (each group contains n=6-15) mice were treated with saline which did not have a preference score. Four groups animals received four different doses of IBNtxA and other four groups mice received four doses of morphine. The initial preference was for three sessions and then training for 10 sessions after that CPP expression for just single session and every session of each stage was for 30 mins. The result confirmed that IBNtxA (green color) did not have a preference when was compared with saline's and morphine's (red color) preference score. The score was calculated by subtracting the pre-training preference score (drug-paired chamber) from the preference score (drug-paired chamber) of the post-training test. Data are presented as means \pm SEM. * $p < 0.05$, repeated-measures 2-way ANOVA with Sidak's multiple comparisons test.

IBNtxA pretreatment reduces place preference for 10 mg/kg morphine

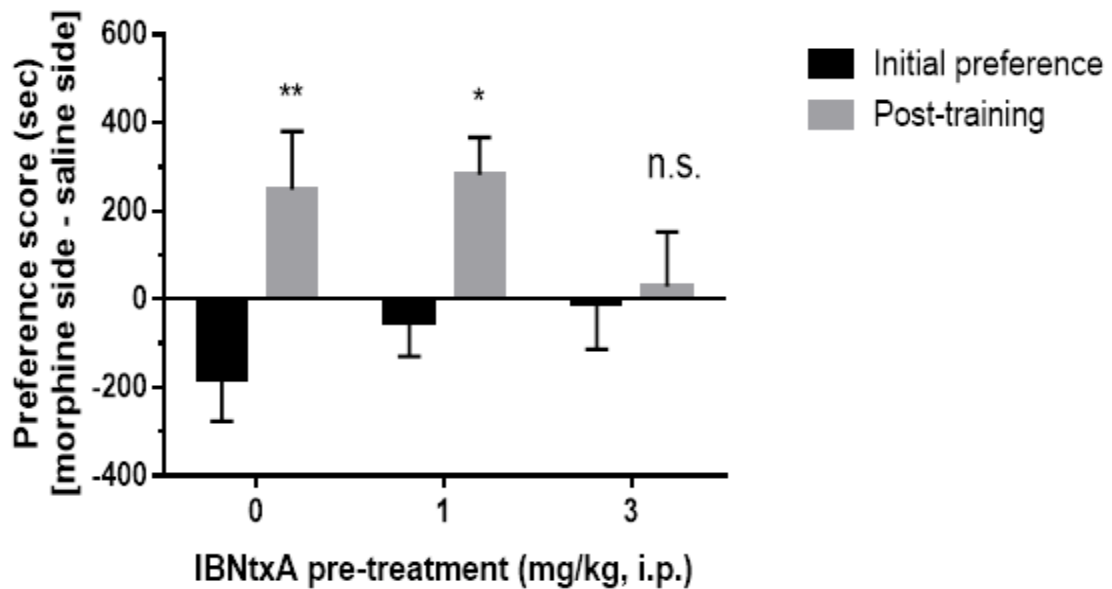


Figure 116. The effect of IBNtxA on morphine-induced place preference. Three groups of animals (n=10-11 per group) which were pretreated with DMSO vehicle, 1 mg/kg IBNtxA, or 3 mg/kg IBNtxA prior to every 10 mg/kg morphine administration during a 10-day training regimen. After training, CPP expression was measured in a 30-minute session in which animals had free access to both drug- and vehicle-paired chambers. 1 mg/kg IBNtxA did not reduce morphine-induced CPP, but mice pre-treated with 3 mg/kg IBNtxA prior to receiving 10 mg/kg morphine showed no morphine place preference. These results indicate that 3 mg/kg IBNtxA attenuated the morphine-induced CPP expression. The data are presenting with means \pm SEM. * $p < 0.05$, repeated-measures 2-way ANOVA with Sidak's multiple comparisons test.

3 mg/kg IBNtxA suppresses hyperlocomotion induced by 10 mg/kg morphine

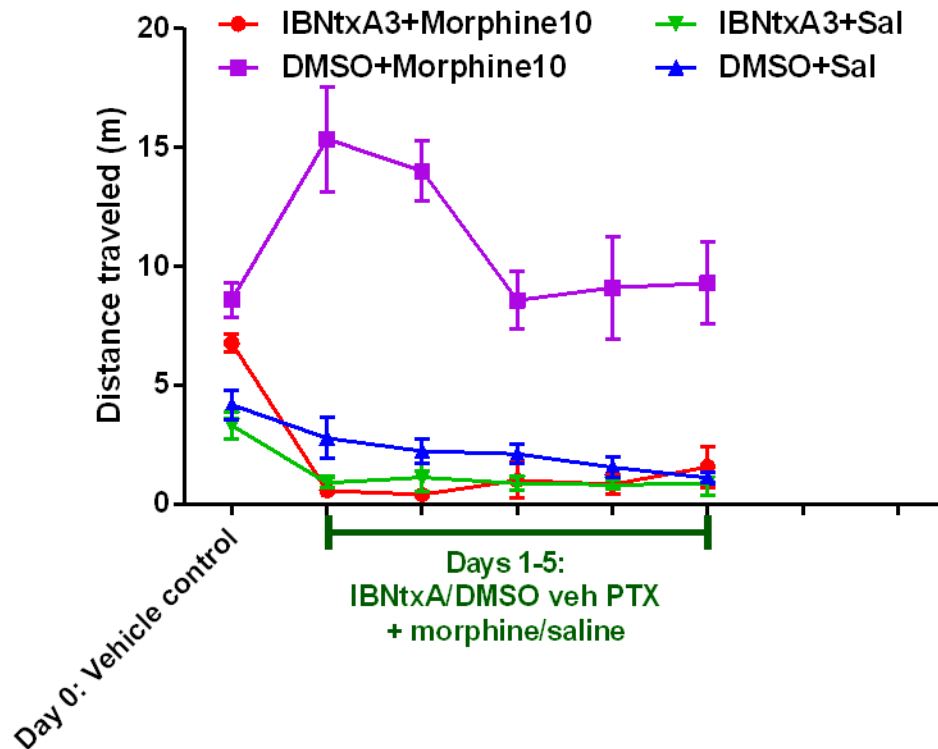


Figure 12. Drugs and/or vehicle-induced locomotor activity. Four groups of animals (n= 6-11 per group) which among two groups of them were pre-treated with 3 mg/kg IBNtxA before getting post-injection with 10 mg/kg morphine and another group with saline. Other two groups were pretreated with DMSO prior to receive morphine 10 mg/kg or saline. Among them animals, received IBNtxA and Morphine traveled significantly less distance than the animals which received DMSO vehicle before getting the same dose of morphine which indicated IBNtxA can suppress morphine-induced hyperlocomotion. Data are presented as means \pm SEM.

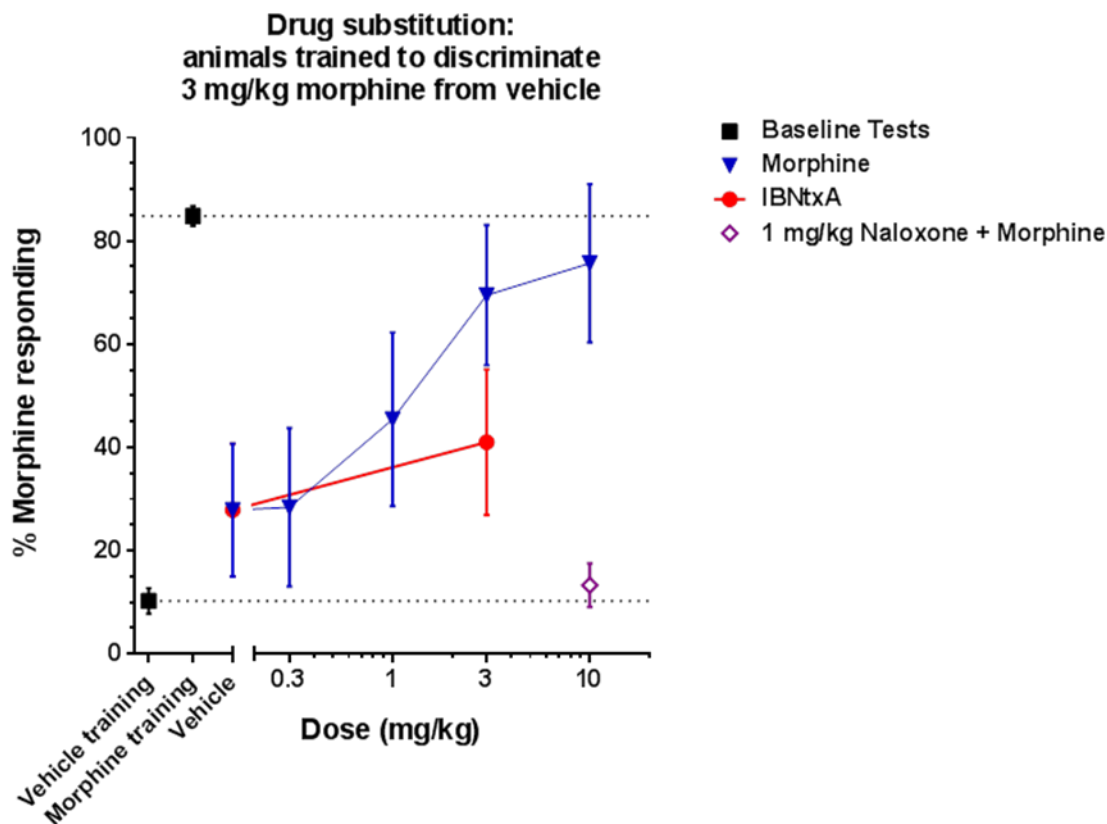


Figure 18. Drug discrimination study with morphine-trained animals. CD-1 outbred mice (n=8) were trained for almost 60 sessions and the duration of each session was an hour and 10±5 mins. The well-trained animals with more than 80% correct response, were tested for discrimination among vehicle, morphine (0.3, 1, 3, and 10 mg/kg) and novel drug IBNtxA 3 mg/kg, the result showed that IBNtxA was a partial substitution of morphine. Pre-treatment (i.p.) with naloxone 1 mg/kg prior to injecting morphine, showed that the morphine response is as lower as a percent of vehicle training responses to morphine side. This is obvious that antagonist naloxone blocked the morphine. The percent of morphine responding was calculated by taking the ratio of morphine responses over total responses. Data presented as means ± SEM.

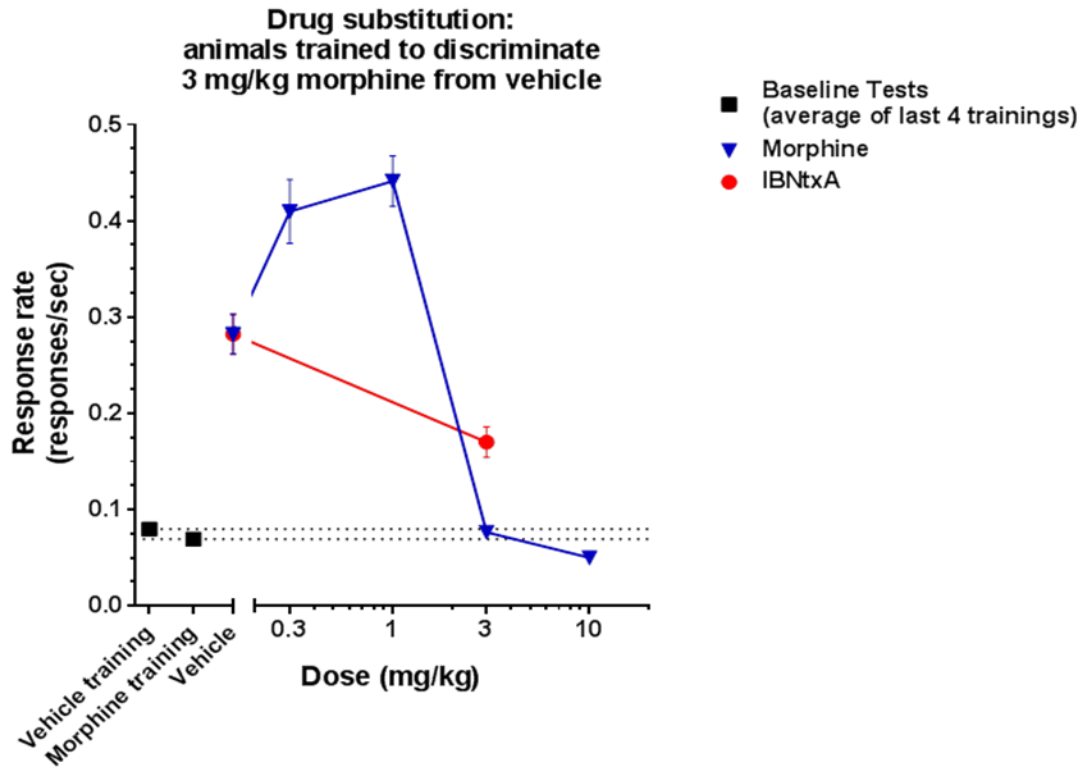


Figure 1913: Rate effects of drug doses in the drug discrimination study with morphine-trained animals. CD-1 outbred mice (n=8) were trained for almost 60 sessions and the duration of each session was an hour and 10±5 mins. The well-trained animals with more than 80% correct response, were tested for discrimination among vehicle, morphine (0.3, 1, 3, and 10 mg/kg) and novel drug IBNtxA 3 mg/kg, the result showed that IBNtxA was a partial substitution of morphine. The rate of responding was calculated as:

$$\text{Response rate} = \frac{\text{Total number of responses}}{\text{Total time (maximum 60 mins)}}$$

Data presented as means ± SEM.

Chapter 3

***Mycobacterium vaccae* Immunization for Drug Addiction, Relapse, and Withdrawal**

Background

Addiction is a major health issue which brings upon other health complications with economic burdens. According to the 2016 National Survey on Drug Use and Health, around 1.7 million people misused stimulant drugs in the U.S.A. and 2.2 percent of them are young adults.(Center, 2011) In this survey, it has been reported that almost 1.9 million people were current cocaine users. Psychostimulants promote dopamine pathways by signaling in the nucleus accumbens, the reward producing area, which calls upon euphoria feelings that eventuate the drug-taking and -seeking and leads to addiction. (Nestler, 2005) Furthermore, long-time cocaine exposure causes neuroinflammation which is another underlying reason for cocaine addiction. The consequences of physiological disorders because of cocaine use are—physical withdrawal, increasing use with time, and failure to participate in works at work, school, or home.

Unfortunately, there is no current FDA-approved treatment for addiction to psychostimulant substances and finding any medicine to prevent addiction and relapse is preeminent for America as well as the rest of the world.

Mycobacterium vaccae

The aims of our collaborative research were in finding new medicine for drug treating addiction, relapse, and withdrawal. Our research was designed based on some unique neuropharmacological properties of *M. vaccae* which is a nonpathogenic environmental bacterium, belongs to the family of Mycobacteriaceae that is found in the soil. Multiple studies have proved that *M. vaccae* has positive effects on certain

neurological disorders such as post-traumatic stress. Immunization with heat-killed *M. vaccae* has immunoregulatory properties and can prevent stress-induced spontaneous colitis, over neuroinflammation, also it has both anxiolytic or fear-reducing effects. (Reber et al., 2016) It works by stimulating the neurons which contain serotonin signaling pathways and heightens the serotonin levels in the dorsal raphe nucleus to respond to stress and anxiety-like behavioral. (Lowry et al., 2007)

Based on *M. vaccine's* pharmacological profile—it might have effects against cocaine addiction and relapse and we studied the effect of *M. vaccae* immunization on cocaine-induced CPP and stress-induced reinstatement of cocaine CPP.



Figure 140. (Bristol, 2007) Colonies of *Mycobacterium vaccae* which was Sauton's agar. (Bristol, 2007) This bacterium is usually found in soil, which is not harmful to human; was isolated in Uganda and that it showed immunization effect against leprosy. After that numerous studies indicated its medical value as a vaccine for different diseases. (Wallis & Johnson, 2009)

Methodology

Animal and drug. Drug and Animal: C57 BL/6 male mice were used for this investigation and as drugs cocaine 30 mg/kg, heat-killed *M. vaccae* (0.1 mg, s.c.) or vehicle (borate-buffered saline) were administered according to the research plan. *M. vaccae* (3 x 0.1 mg, s.c.), regimen is reliable in both mice and rat models for neurological and behavioral studies.

General cocaine CPP procedure. The CPP training for cocaine is almost the same as mentioned in the early in methodology session. But prior to providing cocaine CPP training, two groups of animals—one group was preimmunized *M. vaccae* (3 x 0.1 mg, s.c.) and another group was preimmunized with vehicle (s.c. borate-buffered saline). To confirm the proper preimmunization, each animal had an individual animal ID to identify the animal which received the vehicle and *M. vaccae*. The immunization was occurred inside the CMRSU vivarium three times over 14 days with even time intervals.

When immunization was done then the cocaine CPP procedure was started which also followed an unbiased procedure where any animal showing >70% initial preference for any compartment was excluded before further training and the duration of training or test session was also for 30 minutes. The qualified animals were then trained over 10 days, either after getting cocaine or saline vehicle injections (i.p.). After 10 days of consecutive training, animals were tested for CPP expression to know the preference score for cocaine-paired side and whether preimmunization did affect the cocaine-induced CPP or not.

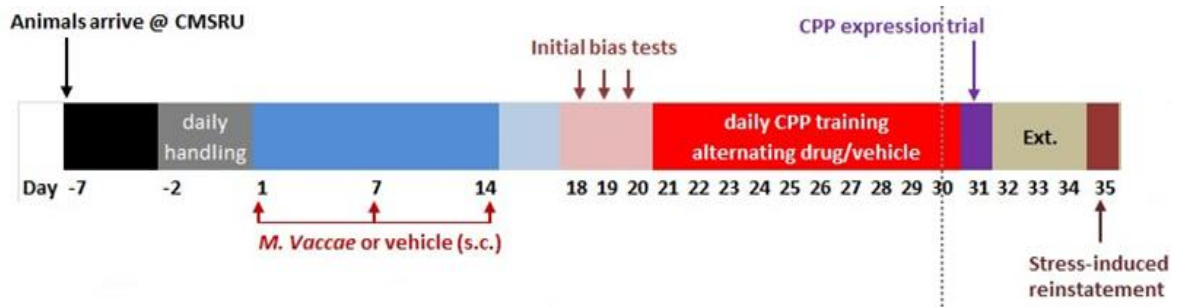


Figure 151. The schematic presentation of *M. Vaccace* preimmunization test for cocaine CPP methodology. The animals arrive in vivarium of Cooper Medical School of Rowan University (CMSRU) where they have been kept for five days. Prior to inject (s.c.) *M. vaccae* (3 x 0.1 mg) or vehicle (borate-buffered saline) three times in 14 days, they were handled for two days to make them comfortable with researchers. After immunization, they are being placed in CPP chambers for initial bias test for three sessions and after that ten days (ten sessions) CPP training. After successfully completing training, they have been tested for CPP expression and finally stress-induced (forced swim) reinstatement test followed by CPP extinction.

Then after two sessions of extinction, we tested the for stress-induced cocaine reinstatement. We used the forced swim method to induce stress prior to placement in CPP chamber which has been detailed earlier.

Statistical Analysis.

During CPP expression

Preference Score

= time spent in drug side on test day

– time spent in drug side before training

During reinstatement test

Preference Score = time spent on drug side – time spent in the vehicle side

Result: *M. vaccae* attenuated stress-induced cocaine CPP

Our investigation found that the animals which were preimmunized with heat-killed *M. vaccae* did not affect the 30 mg/kg cocaine-induced CPP immediately after training and it was near same as vehicle immunized animals. But the in stress-induced reinstatement, there was significant changed between the vehicle and *M. vaccae* group. Heat-killed *M. vaccae* preimmunized animals showed almost no preference for the cocaine-paired side but the animals which preimmunized with vehicle scored higher. This of result indicated that though *M. vaccae* did not affect the cocaine-induced CPP after training it effectively attenuated the stress-induced cocaine CPP.

Discussion

Relapse is the most unavoidable event during the addiction treatment one of the principal reasons for relapse is stress. (Sinha, Garcia, Paliwal, Kreek, & Rounsaville, 2006) Scientists often test this human stress-induced relapse in animals through stress-induced reinstatement. (Y. Shaham, U. Shalev, L. Lu, H. de Wit, & J. Stewart, 2003) Like any other addiction treatment, cocaine relapse is also challenging to fight. Heat-killed *M. vaccae* preimmunization clearly reduced the effects of stress-induced relapse for cocaine addictive animals (*Figure 22*). Since *M. vaccae* also has anxiolytic effects, and our investigation proved the anti-relapse effects, it might be beneficial for the patients who are in treatment for cocaine addiction.

Conclusion

Finding a new medicine for treating cocaine addiction is intriguing but at the same time one of demanding job. Our current umbrella project for finding a medicine for neuropsychiatric disorders including addiction, abuse and dependence is continuing investigation with new idea and with new collaborations. Investigations on properties of *M. vaccae* are happening now with some specific set of goals which will soon decode more addiction neuropharmacological properties of this nonpathogen bacterium along with the positive effects on stress-induced reinstatement for cocaine.

***M. vaccae* pretreatment significantly attenuates stress-induced reinstatement of place preference for 30 mg/kg cocaine**

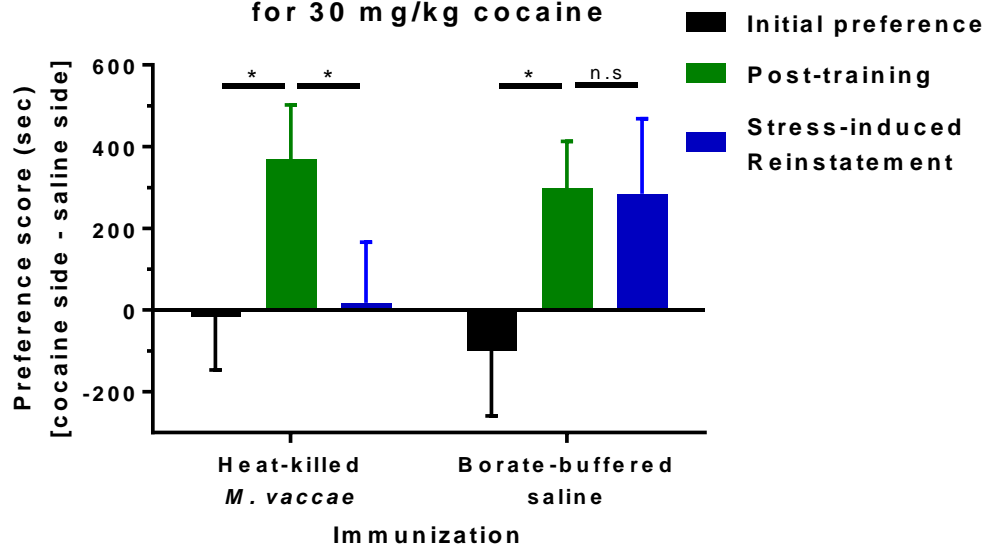


Figure 162. M. vaccae preimmunization effects on cocaine CPP. Two groups of animals (n=8-12 for each group) were used for this test. Animals were pre-immunized with vehicle or heat-killed *M. vaccae* three times in seven days intervals and then were palced for three initial preference test , 10 sessions of CPP training with cocaine 30 mg/kg. After training animals were tested for CPP expression and then, stress-induced (forced-swim) reinstatement followed by extinctions. Animals nevertheless immunization with *M. vaccae* or vehicle, did not alter cocaine 30 mg/kg induced CPP acquisition wherein both vehicle and heat-killed *M. vaccae* (3 x 0.1 mg, s.c.) showed the considerable score for the cocaine-paired side. But after the extinction sessions, stress-induced (forced-swim) reinstatement was attenuated by heat-killed *M. vaccae* preimmunization. Data presented as means \pm SEM. *p < 0.05, repeated-measures 2-way ANOVA with Sidak's multiple comparisons test.

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