Characterization of novel $\alpha_2/\alpha_3$ subtype-selective GABA$_A$ receptor positive allosteric modulators

by

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January 14, 2019

A dissertation submitted to the faculty of the Graduate School of the University at Buffalo, The State University of New York in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Neuroscience Program
Jacobs School of Medicine and Biomedical Sciences
The State University of New York at Buffalo
Acknowledgments

First, my sincere gratitude goes to my research advisor and mentor Dr. Jun-Xu Li. The work presented in this dissertation would not have been possible if it were not for him. When I first entered Dr. Li’s lab, I knew very little about behavioral pharmacology. He taught me so much about pharmacology and experimental design. I truly appreciate his patience, wisdom, mentorship, passion, and encouragement. Most of all, I admire and appreciate his work ethic. Whether it was a weekend, a holiday, or a snow storm, Dr. Li made a great effort to be in the laboratory. It was truly an honor being your trainee and I plan to take all that I learned from you with me throughout my career.

Next, I would like to thank my dissertation committee, Dr. Arin Bhattacharjee, Dr. Derek Daniels, and Dr. David Dietz, and my program director Dr. Malcolm Slaughter. Thank you for your advice, encouragement, and guidance throughout my training. I appreciate your support with everything from writing recommendation letters to providing me with valuable feedback at poster presentations. I would especially like to thank Dr. Slaughter and Dr. Dietz for their support and assistance with the Neuroscience Graduate Student Association. Additionally, I would like to thank Dr. Caroline Bass for her encouragement and career advice throughout my training.

I would also like to thank Dr. Margarita Dubocovich, Dr. Raj Rajnarayanan, past and current coordinators of the Institute for the Strategic Enhancement of Educational Diversity (iSEED) program and the Initiative for Maximizing Student Development (IMSD) program for providing me with resources and professional development workshops during my training.

Then, I would like to thank the laboratory of Dr. James M. Cook at the University of
Wisconsin-Milwaukee for providing me with the compounds that allowed me to carry out my dissertational studies. The experiments discussed in this dissertation would not have been possible without them.

To say I have the best lab mates in the world would be an understatement. To the past and current members of the Li lab, I am so grateful to have worked with you all. I have learned so much from all of you. Thank you for your advice, support, encouragement, and all the laughs we had along the way #LiLabStrong!

Lastly, I would like to thank my family and friends for their continual love and support. I would not have made it without you. I am forever grateful for your friendship, encouragement, and prayers throughout the years. To my parents, I appreciate the sacrifices that you both made to get me to this point. Thank you for instilling values in me that I will carry on throughout the rest of my life. Thank you for teaching me the importance of education, diligence, hard work, and faith.
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ABSTRACT

Millions of individuals endure persistent pain. In the United States alone, about 30% of the population is estimated to suffer from chronic pain. In addition to the physical and emotional burden that chronic pain imparts, chronic pain represents a financial challenge, costing the nation up to $635 billion in treatment and lost productivity. Currently, the most widely used analgesics include µ-opioid agonists, anti-inflammatory steroids, and nonsteroidal anti-inflammatory drugs (NSAIDs). While these analgesics are useful in some pain conditions, their use is limited due to lack of efficacy and various side-effects. Thus, the development of novel and effective analgesics remains a clinical need.

Spinal GABA\(_A\) receptors are thought to play a key role in pain processing. Previous studies have shown that local spinal application of benzodiazepine site ligands (that positively modulate GABA\(_A\) receptor function) alleviates pain in both neuropathic and inflammatory pain models. Thus, GABA\(_A\) receptors have received increasing attention as a promising target for the development of novel analgesics. Point mutation studies have aided in dissecting which GABA\(_A\) \(\alpha\)-subtypes mediate different effects of benzodiazepines. The \(\alpha2\) and \(\alpha3\) subtypes of GABA\(_A\) receptors are associated with the analgesic effects of benzodiazepines. These studies have led to the development of novel subtype-selective GABA\(_A\) positive allosteric modulators (PAMs). However, very few of the PAMs that are reported are truly selective for solely \(\alpha2\) and \(\alpha3\) subtypes. Therefore, much remains unknown regarding the full therapeutic potential of \(\alpha2/\alpha3\) subtype-selective GABA\(_A\) PAMs.

Firstly, it was unknown whether \(\alpha2/\alpha3\) subtype-selective GABA\(_A\) PAMs were truly effective for attenuating pain due to their lack of \(\alpha5\) activity (a subtype that was initially thought
to be necessary to produce analgesic effects). The studies presented in chapter 2 demonstrate that α2/α3 subtype-selective GABA<sub>A</sub> PAMs can produce robust analgesic effects, devoid of tolerance, in two different models of chronic pain. Additionally, α2/α3 subtype-selective GABA<sub>A</sub> PAMs can attenuate acute pain depending on the behavioral output and the degree of pain.

Secondly, it was unknown whether GABA<sub>A</sub> PAMs and opioids were suitable for combination therapy. The studies done in chapter 3 demonstrate that α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs KRM-II-81 and NS16085 produced mainly additive and some supra-additive effects when combined with either oxycodone or morphine in CFA-treated rats (as demonstrated in the mechanical nociception assay). There were no infra-additive (antagonistic) effects observed in any of the fixed ratios between the GABA<sub>A</sub> PAMs and opioids.

Thirdly, previous studies have shown that PAMs that are selective for α2/α3/α5 subtype-containing GABA<sub>A</sub> receptors have the ability to produce analgesia devoid of other undesired effects. The relationship between the analgesic effects and other off-target pharmacological effects of α2/α3 subtype-selective GABA<sub>A</sub> PAMs remained underexplored. The studies presented in chapter 4 demonstrated that while the analgesic effects of the nonselective midazolam were masked by other side-effects, α2/α3 subtype-selective GABA<sub>A</sub> PAMs were able to selectively produce analgesic effects, devoid of side effects such as sedation and cognitive impairment. Overall, these studies further support the clinical potential of α2/α3 subtype-selective GABA<sub>A</sub> PAMs as potential analgesics.
CHAPTER 1 – BACKGROUND

Chronic Pain: a clinical challenge

More adults in the United States are affected by chronic pain than cancer, heart disease, and diabetes combined (IOM, 2011). Chronic pain imposes not only physical and emotional discomfort on its sufferers, but represents a national challenge, costing the nation up to $635 billion in treatment and lost productivity in 2010 (Gaskin & Richard, 2012). Whether the etiology is inflammatory or neuropathic in origin, chronic pain is commonly characterized by heightened sensitivity, increased response to noxious stimuli (hyperalgesia) and pain produced by normally non-noxious stimuli (allodynia) (Jensen & Baron, 2003). Currently, opioids are the standard for chronic pain management. Although, opioids are effective for treating chronic pain, they have detrimental properties that limit their use. Opioids are associated with tolerance, abuse liability, respiratory depression, and constipation (Dumas & Pollack, 2008; Ling, Mooney, & Hillhouse, 2011). Therefore, the development of novel analgesic compounds remains a clinical need. Despite progress in the understanding of the physiological and underlying mechanisms of pain, there are few novel analgesics that have made it into clinical trials.

Loss of GABA-mediated inhibition after pain

Advances in pain research has increased our understanding of the underlying mechanisms of the pathophysiology of chronic pain. The notion that inhibitory neurons serve a critical function in spinal pain control was first proposed in the gate control theory of pain. According to the gate control theory of pain, the transmission of pain signals (nociceptors) from the periphery
to the spinal cord is modulated by both excitatory and inhibitory neurons emanating from the brain (Melzack & Wall, 1965). The primary inhibitory neurotransmitter in the central nervous system is gamma-aminobutyric acid (GABA). Chronic pain states are associated with a reduced GABA-mediated inhibitory function (Chesnut et al., 1996). When animals are injected intrathecally with GABA_A receptor antagonists, hyperalgesia is observed (Roberts, Beyer, & Komisaruk, 1986). Thus, it is theoretically plausible to reverse the GABAergic disinhibition by enhancing GABAergic function, which should lead to analgesia. This theory has been further studied in rodent models of chronic pain. Previous studies have shown that local spinal application of benzodiazepine site ligands (that positively modulate GABA_A receptor function) alleviates pain in both neuropathic and inflammatory pain models (Knabl et al., 2008; Ralvenius, Benke, Acuna, Rudolph, & Zeilhofer, 2015). As a result, spinal GABA_A receptors have received increasing attention as a suitable pharmacological target for pain management.

**GABA_A receptors: Structure, Function, and Modulation**

GABA_A receptors mediate many of the physiological actions of GABA. These receptors are ligand-binding channels that consist of five subunits (typically comprising 2α, 2β and 1γ subunits) surrounding a chloride-ion conducting pore. GABA_A receptors are activated once an agonist such as GABA is bound. These receptors can be modulated by a variety of drugs such as benzodiazepines, neurosteroids, barbituates, convulsants, and anesthetics (Sieghart, 2006). In addition, positive allosteric modulators (PAMs) can also modulate GABA_A receptor activity. PAMs of the GABA_A receptor act on the benzodiazepine site (located at the α/γ subunit interface) of GABA_A receptors in the presence of an agonist. High affinity binding of PAMs to their recognition site, brings about a conformational change in the receptor such as to increase the affinity for channel gating by GABA.
Benzodiazepines are a class of GABA\textsubscript{A} receptor PAMs that are widely used for a number of clinical conditions. At the molecular level, benzodiazepines bind to and interact with four different \(\alpha\) subtypes (\(\alpha1\), \(\alpha2\), \(\alpha3\), \(\alpha5\)). Point mutation studies and other studies employing pharmacological approaches have shown that different \(\alpha\)-subtypes mediate different pharmacological actions of benzodiazepines (Crestani & Rudolph, 2015). For example, the sedative and abuse-related effects of benzodiazepines are primarily mediated through \(\alpha1\) subtypes, the analgesic and anxiolytic effects are thought to be mediated through \(\alpha2/\alpha3\) subtypes, and cognitive functions through \(\alpha5\) subtypes of GABA\textsubscript{A} receptors. Therefore, due to activity at \(\alpha1–3, \alpha5\) subtypes of the GABA\textsubscript{A} receptor, benzodiazepines are not suitable as analgesics. It is likely that the analgesic effects of benzodiazepines are masked by other pharmacological effects that are unrelated to analgesia, such as sedation (Ralvenius et al., 2015). This is most likely due to the expression of the different \(\alpha\) subtypes within the central nervous system. While \(\alpha2/\alpha3\) subtype-containing GABA\textsubscript{A} receptors are located in brain regions such as the hypothalamus, amygdala, and medulla; they are mainly concentrated in dorsal horn of the spinal cord (Sieghart, 2006). Additionally, \(\alpha1\) subtype containing GABA\textsubscript{A} receptors are the most abundant GABA\textsubscript{A} receptors in the brain. This is why benzodiazepines have been shown to alleviate pain when injected directly into the spinal cord, but not systemically (Nishiyama, 2006). Developing \(\alpha2/\alpha3\) subtype-selective GABA\textsubscript{A} receptor PAMs may be a suitable pharmacological approach to restore diminished inhibitory spinal pain control, thus managing pain.

*Subtype-selective GABA\textsubscript{A} PAMs in rodent models of pain*

Point mutation studies have aided in dissecting which GABA\textsubscript{A} \(\alpha\)-subtypes mediate different effects of benzodiazepines. In addition, these studies have led to the development of novel subtype-selective GABA\textsubscript{A} PAMs. The majority of reported subtype-selective PAMs are...
selective for α2, α3, and α5 subtypes of the GABA_A receptor. For example, L-838417 (7-tert-butyl-3-(2,5-difluoro-phenyl)-6-(2-methyl-2H-[1,2,4] triazol-3-ylmethoxy)-[1,2,4]triazolo[4,3-b]pyridazine) has a selectivity profile of α2=α3 = α5 > α1 (R. M. McKernan, Rosah., T.W., Reynolds, D.S., Sur, C., Wafford, K.A., Atack, J.R., Farrar, S., et al., 2000). This compound has been shown to be effective for attenuating inflammatory and neuropathic pain, all the while devoid of unwanted sedation, motor impairment, and tolerance development (Knabl et al., 2008). Another, reported subtype-selective GABA_A PAM is NS11394 (3’[5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile) has a selectivity profile of α5>α3>α2>α1 (Mirza et al., 2008). NS11394 has been shown to attenuate spontaneous nociceptive behaviors in formalin- and capsaicin-induced pain states, attenuate ongoing nociception in both inflammatory and neuropathic pain models. Another reported subtype-selective GABA_A PAM HZ166 (α3=α2>α5=α1), has been shown to produce antiseizure activity, and produced antinociceptive effects in mice with zymosan A-induced inflammation and CCI-induced neuropathic pain (Di Lio et al., 2011).

While L-838417, NS11394, and HZ166, have been shown to produce analgesic effects. One issue is that the majority of these reported subtype-selective GABA_A PAMs display efficacy at α5-subtype containing GABA_A receptors. This presents a problem because of the proposed role of α5 subtypes of the GABA_A receptor. Since, α5 GABA_A receptor negative allosteric modulators (NAMs) enhance cognition, PAMs selective for α5-subtypes could possibly impair cognition (Atack, 2010). In fact, both NS11394 and L-838417 have been shown to strongly impair memory in the contextual fear paradigm (measuring negative association of
context), and in the Morris water maze (measuring spatial learning) (Hofmann et al., 2012). This is most likely due to their efficacy at α5-subtype containing GABA<sub>A</sub> receptors.

As a result, recent efforts have focused on developing GABA<sub>A</sub> PAMs with efficacy at α2/α3-subtype containing GABA<sub>A</sub> receptors. Ultimately, compounds that are truly selective for α2/α3 subtype-containing GABA<sub>A</sub> receptors are scarce and only a few have been reported. Our lab reported a novel selective α2/α3-subtype selective GABA<sub>A</sub> PAM KRM-II-81 (5-(8-ethynyl-6-(pyridine-2-yl)-4Hbenzo[f]imidazo[1,5-a][1,4]diazepin-3-yl)oxazole) (Lewter et al., 2017). De Lucas et al., were the first to report a novel α2/α3 subtype-selective GABA<sub>A</sub> PAM NS16085 (4-chloro-3-{6-[5-(2-hydroxypropan-2-yl)-1H-1,3-benzodiazol-1-yl]pyridin-2-yl}benzonitrile) (de Lucas et al., 2015). Recently, Fischer et al., described the novel GABA<sub>A</sub> PAM MP-III-024 (methyl 8-ethynyl-6-(pyridine-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate) (Fischer et al., 2017). Since, very few of the PAMs that are reported are truly selective for solely α2 and α3 subtypes, much remains unknown regarding the full therapeutic potential of α2/α3 subtype-selective GABA<sub>A</sub> PAMs.

This dissertation aims to study the efficacy, interaction with opioids, and the side-effect profile of novel α2/α3 subtype-selective GABA<sub>A</sub> receptors PAMs, to test the analgesic potential of these compounds. Prior to the start of the studies outlined in this dissertation, the analgesic profile of α2/α3 subtype-selective GABA<sub>A</sub> PAMs (devoid of α5 selectivity) had not been reported. Therefore, the analgesic effects of true α2/α3 subtype-selective GABA<sub>A</sub> PAMs were unknown. The first aim of this dissertation was to further investigate the analgesic potential of α2/α3 subtype-selective GABA<sub>A</sub> PAMs in animal models of acute and chronic pain. First, this study sought to examine the analgesic effects of α2/α3 subtype-selective GABA<sub>A</sub> PAMs in a model of
acute visceral pain. Additionally, this visceral pain model was also used to study the ability of α2/α3 subtype-selective GABA_A PAMs to restore pain-depressed behavior. A concept that has not yet been explored with subtype-selective GABA_A PAMs. Additionally, GABA_A PAMs that are truly selective for α2/α3-subtypes, had not been studied in animal models of chronic pain. Thus, this dissertation sought to study the duration of action of α2/α3 subtype-selective GABA_A PAMs to produce analgesia. Lastly, while repeated administration of α2/α3/α5 subtype-selective GABA_A PAMs have not displayed tolerance to the analgesic effects, tolerance development has not been studied with α2/α3 subtype-selective GABA_A PAMs prior to the work outlined in this dissertation.

The next aim of this dissertation was to determine whether α2/α3 subtype-selective GABA_A PAMs are well suited to be used in combination therapy. Combination therapy, combining more than one drug in a treatment regimen, has been used in various clinical areas such as asthma, oncology, and hypertension (Juniper, Jenkins, Price, & James, 2002; Law, Wald, Morris, & Jordan, 2003). Although monotherapies are effective under some conditions, there are some cases in which combination therapy has been shown to be more effective. The goal of combination therapy is to achieve the same or better analgesic effects while reducing undesired side effects that are likely to result from higher doses of a single drug (Gilron, Jensen, & Dickenson, 2013). Combination pharmacotherapy for pain control remains an important and understudied strategy. It has not been determined whether α2/α3 subtype-selective GABA_A PAMs and opioid co-treatment produces additive, infra-addictive, or supra-additive effects. Therefore, we sought to study the drug interactions of GABA_A PAMs, midazolam, KRM-II-81 and NS16085 and opioids oxycodone and morphine.
The final aim of this dissertation was to further characterize $\alpha_2/\alpha_3$ subtype-selective GABA$_A$ PAMs using a series of in vivo studies to confirm $\alpha_2/\alpha_3$ subtype-selectivity. There is considerable evidence implicating that different $\alpha$ subunits of the GABA$_A$ receptor are associated with different actions of the benzodiazepine site of the GABA$_A$ receptor. The $\alpha_1$ subunit has been shown to mediate sedative and abuse-related effects, $\alpha_2/\alpha_3$ subunits are thought to mediate analgesic, anxiolytic, and myorelaxant effects, and the $\alpha_5$ subunit has been associated with cognition (Zeilhofer, Ralvenius, & Acuña, 2015). As a result, attempts have been made to develop subtype-selective GABA$_A$ PAMs in order to manage various clinical areas. The relationship between the antinociceptive effects and other off-target pharmacological effects of $\alpha_2/\alpha_3$ subtype-selective GABA$_A$ PAMs remains underexplored. Therefore, this dissertation sought to examine some behavioral effects and the selectivity profile of $\alpha_2/\alpha_3$ subtype-selective GABA$_A$ PAMs.
CHAPTER 2A – ANTINOCICEPTION IN ACUTE PAIN

INTRODUCTION

There have been no mechanistically novel analgesics developed in the past 50 years (Kissin, 2010). Some argue that the low success rate in the discovery of new analgesics could be partially due to the poor translation of existing animal models of pain (Burma, Leduc-Pessah, Fan, & Trang, 2017; Negus et al., 2006). For example, most studies of pain only use pain-stimulated behaviors such as tail flick or paw withdrawal. Expanding the range of pain models used in preclinical studies to include pain-depressed behaviors may help to discover analgesics with better clinical success, and may also have higher face validity with respect to clinical pain conditions (e.g., chronic pain sufferers likely move less to avoid aggravating their pain as opposed to reacting more strongly to external noxious stimuli) (Negus et al., 2006).

Emerging evidence support the notion that developing α2/α3 subtype-selective GABA_A receptor PAMs may be a strategy to discover novel analgesics. Previous studies have identified a quite selective moderate-to-low efficacy α2/α3 subtype-selective GABA_A receptor PAM NS16085, which demonstrated partial suppression of nociceptive behaviors in the formalin assay (de Lucas et al., 2015). In an effort to develop novel α2/α3 subtype-selective GABA_A receptor PAMs, we report a new compound, KRM-II-81 (5-(8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-3-yl) oxazole) (Figure 1), which demonstrated the profile as a highly selective α2/α3-specific GABA_A receptor PAM. We first examined the α subtype specificity of KRM-II-81 using electrophysiological recording in cells expressing different α subtype GABA_A receptors. We then examined the antinociceptive effects of KRM-II-81 in several mice models of chemical stimulation induced visceral pain. Chemical-induced pain
models were chosen because previous studies showed that they are sensitive to pharmacological modulation of GABA_A receptors (Chiba, Nishiyama, Yoshikawa, & Yamada, 2009; Knabl et al., 2008). A structurally similar compound, KRM-II-18B (5-(8-ethynyl-6-(2-fluorophenyl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-3-yl) oxazole) (a non-selective GABA_A receptor PAM) was also studied in parallel for comparison. In addition to chemical-induced pain models, the acute antinociceptive effects of KRM-II-81 were also observed using the warm water tail-flick assay.
CHAPTER 2A – ANTIMOCICEPTION IN ACUTE PAIN

MATERIALS AND METHODS

**Cellular studies:** Transfection of mammalian cells and electrophysiological recordings

Full-length cDNAs for GABA\(_A\) receptor subtypes (generously provided by Dr. Robert Macdonald, Vanderbilt University and Dr. David Weiss, University of Texas Health Sci. Center, San Antonio TX) in mammalian expression vectors were transfected into the human embryonic kidney cell line HEK-293T (GenHunter, Nashville, TN). All subtypes were rat clones except for \(\alpha_2\), which was a human clone. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal bovine serum, 100 IU/ml penicillin and 100 \(\mu\)g/ml streptomycin.

HEK-293T cells were transiently transfected using calcium phosphate precipitation. Plasmids encoding GABA\(_A\) receptor subtype cDNAs were added to the cells in 1:1:1 ratios (\(\alpha:\beta:\gamma\)) of 2 \(\mu\)g each\(^{31}\). For identification of positively transfected cells, 1 \(\mu\)g of the plasmid pHook™-1 (Invitrogen Life Technologies, Grand Island NY) containing cDNA encoding the surface antibody sFv was also transfected into the cells (Chesnut et al., 1996). Following a 4–6 hr. incubation at 3% CO\(_2\), the cells were treated with a 15% glycerol solution in BBS buffer (50 mM BES(N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na\(_2\)HPO\(_4\)) for 30 sec. The selection procedure for pHook expression was performed 18–52 hrs later. The cells were passaged and mixed for 30–60 min. with 3–5 \(\mu\)l of magnetic beads coated with antigen for the pHook antibody (approximately 6 \(\times\) 10\(^5\) beads) (Chesnut et al., 1996). Bead-coated cells were isolated using a magnetic stand. The selected cells were resuspended into supplemented DMEM, plated onto glass coverslips treated with poly L-lysine and collagen, and used for recordings the next day. Cells were patch-clamped at −50 mV in the whole-cell recording.
configuration. The bath solution consisted of (in mM): 142 NaCl, 8.1 KCl, 6 MgCl₂, 1 CaCl₂, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. Recording electrodes were filled with a solution of (in mM); 153 KCl, 1 MgCl₂, 5 K-EGTA (ethylene glycol-bis (β-aminoethyl ether N,N,N′N′-tetraacetate), and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295–305 mOsm. GABA was diluted into the bath solution from freshly made or frozen stocks in water. Compounds were dissolved in DMSO and diluted into bath solution with the highest DMSO level applied to cells of 0.01%. Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) on a two-stage puller (Narishige, Japan) to a resistance of 5–10 MΩ. Solutions containing GABA or GABA+ compounds were applied to cells for 5 sec. using a 3-barrelled solution delivery device controlled by a computer-driven stepper motor (SF-77B, Harvard Apparatus, Holliston, MA, open tip exchange time of <50 msec). There was a continuous flow of external solution through the chamber. Currents were recorded with an Axon 200B (Foster City, CA) patch clamp amplifier.

Whole-cell currents were analyzed using the programs Clampfit (pClamp9 suite, Axon Instruments, Foster City, CA) and Prism (Graphpad, San Diego, CA). Concentration-response data was fit with a four-parameter logistic equation (Current = [Minimum current + (Maximum current - Minimum Current)]/1+(10^(log EC₅₀ – log [modulator]))^n) where n represents the Hill number. All fits were made to normalized data with current expressed as a percentage of the response to GABA alone for each cell.

Subjects: Adult male ICR mice (Envigo, Indianapolis, IN, USA) that were 8–12 weeks old and weighed 30–40g upon arrival were used in these studies. Mice were housed in pairs, except those
used in the nesting procedure. Mice were on a 12/12 h reverse light/dark cycle (lights on at 6 PM and off at 6 AM) and had free access to water and food except during experimental sessions. All experiments were performed during the dark cycle. Animals (n = 6–8 per group) were maintained and experiments were conducted in accordance with guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York (Buffalo, NY), and with the 2011 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington, DC).

**Writhing Behavior:** Mice were habituated in a clean mouse cage with corn cob bedding for 20 min. Mice then received intraperitoneal (i.p.) injections of either 0.32% lactic acid or 0.6% acetic acid. The number of acid-induced writhes in a 25 min observation period was recorded, starting 5 min after the injection of acetic acid or 10 minutes after the injection of lactic acid. These starting times and observation period were decided according to a pilot study to ensure that the observation period included the majority of writhing responses. A writhe was defined as a contraction of the abdomen following a stretch of the hind limbs. Mice were randomly selected for treatment groups or vehicle groups. Mice in treatment groups received subcutaneous (s.c.) injections of either morphine (0.1–3.2 mg/kg, 10 min pretreatment), KRM-II-18B (1–10 mg/kg, 30 min pretreatment), or KRM-II-81 (3.2–10mg/kg, 30 min pretreatment). In flumazenil studies, mice received s.c. injections of flumazenil 15 minutes prior to injections of each PAM.

**Nesting Behavior:** Singly housed mice were tested in their home cages with familiar (≥ 2 days of habituation) corncob bedding (parameters established during pilot studies according to
previous study (Negus et al., 2015). Mice were assigned to a particular dose of a treatment drug. Before the start of each test session, mice were acclimated to the procedure room for at least 30 min. Mice received pretreatments of vehicle, morphine, KRM-II-18B, or KRM-II-81 and were briefly transferred to a second cage while any existing nesting material (VWR Scientific, Randor, PA, USA) was removed from the home cage. Then, six 2 × 3 cm pieces of new nesting material were distributed around the home cage, each marking a designated zone within the cage. Mice received i.p. injections of either acetic or lactic acid, then returned to the home cage for a 60 min nesting period, during which the cotton pad pieces were retrieved and used to build a nest. At the end of the nesting period, each zone from which a cotton pad piece was retrieved was considered a cleared zone.

**Locomotion Activity:** Locomotor activity was measured using an infrared motion-sensor system (AccuScan Instruments, Inc., Columbus, OH) surrounding Plexiglas cages (40 × 40 × 30 cm). Versa Max software (Omnitech Electronics, Inc., Columbus, OH) was used to monitor the distance the animal travelled for a total of 65 min. Baseline sessions in which mice received s.c. injections of either acetaminophen, KRM-II-18B, or KRM-II-81 and were then placed in the locomotion chamber (n = 6–8 per group) were conducted before test sessions in order to verify that the doses used did not significantly decrease locomotor activity. After 30 minutes in the chamber, mice received i.p injections of vehicle. Test studies were identical except that mice received injections of either 0.6% acetic acid or 0.32% lactic acid instead of vehicle at the 30 min time point. In all sessions, the total distance traveled (cm) by each mouse was used to measure locomotor activity. The 5 minutes immediately following i.p. injection (either vehicle or acid) was excluded from the total distance due to increased locomotion after handling.
Warm water tail-flick assay: The warm water tail withdrawal procedure was conducted as previously described (Li, Becker, Traynor, Gong, & France, 2007; Thorn, Zhang, Peng, Winter, & Li, 2011). For this procedure, two Dual Poly Pro water baths were used (model RS-PB-200; Revolutionary Science, Lindstrom, Minnesota, USA). Each of the water baths consisted of two chambers that contained heated water that was pre-set to the following temperatures; 44°C, 48°C, or 52°C. A multiple-cycle procedure was used to determine the dose-effect curves of the test drugs with an inter-cycle time of 30 min. Briefly, mice were slightly restrained and the tail (~ 2 cm from the tip of the tail) was immersed in the three different warm water baths. The time (sec) in which the tip of the tail was withdrawn from the water bath was defined as the tail withdrawal latency. For each test, tail withdrawal latencies were measured for each of the three temperatures with ~1 min between determinations.

Drugs: KRM-II-18B and KRM-II-81 were obtained from Dr. James M. Cook (University of Wisconsin) according to published procedure (Poe et al., 2016) and dissolved in a vehicle of 20% dimethyl sulfoxide (Amresco, Solon, OH), and 10% emulphor (Solvay, Cranbury, NJ), in 0.9% saline. Morphine sulfate was provided by Research Technology Branch, National Institute and Drug Abuse, National Institutes of Health (Rockville, MD, USA) and was dissolved in 0.9% saline. Acetaminophen was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in a vehicle of 20% dimethyl sulfoxide in 0.9% saline. All drugs were administered subcutaneously. Lactic acid purchased from Sigma-Aldrich was diluted to 0.32% and acetic acid purchased from Macron Fine Chemicals (Center Valley, PA, USA) was diluted to 0.6% in 0.9% saline. Both lactic and acetic acid were administered intraperitoneally.
**Data Analysis**: Statistical analyses were performed with the GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SEM. For studies of writhing, nesting, and locomotor activity, data were analyzed with one-way analysis of variance (ANOVA). Bonferroni’s multiple comparison *post hoc* test was used to determine statistical significance. For the study of the antagonism effects of flumazenil (writhing), data were analyzed with student’s *t*-test. For the warm water tail withdrawal study, tail withdrawal latency was expressed as a percentage of the maximal possible effect (MPE) using the following formula: % MPE = [(test latency – control latency) / (20 s – control latency)] × 100, where the control latency was defined as the latency determined in the absence of drug. Within the dose range studied, neither morphine nor KRM-II-81 produced a well-defined dose-response curve (points ranging from < 50% MPE and > 50%) in 44 °C (all data points were above 50%) and 52 °C (all data points were below 50%). Therefore, only data from 48°C water was used for data analysis. Critical values for the main or interaction effects were set at *p*<0.05.
CHAPTER 2A – ANTINOCICEPTION IN ACUTE PAIN

RESULTS

In vitro Characterization

HEK-293T cells were transiently transfected with one of the six different α subunit subtypes along with the same β (β3) and γ (γ2L) subunits. To determine the sensitivity to modulation, a submaximal concentration of GABA was co-applied with the modulator for 5 sec to cells voltage-clamped at −50 mV. The GABA concentration represented an EC<5 μM for each isoform (Picton & Fisher, 2007) and was 0.1 μM (α6), 0.3 μM (α4, α5), 1 μM (α1, α2) or 3 μM (α3). As expected, receptors containing α4 or α6 subunits were insensitive to a 1 μM concentration of any of these compounds (Figure 2E). KRM-II-18B was an effective and potent modulator of all the benzodiazepine-sensitive receptor isoforms, enhancing the response to GABA to comparable maximum levels and with similar EC50’s (Figure 2A,2C). The average EC50 (and peak response) for potentiation of the response to GABA by KRM-II-18B was 738.8 ± 251.9 nM (410.6 ± 26.3%) for α1β3γ2L (n=7), 261.4 ± 115.8 nM (320.0 ± 14.3%) for α2β3γ2L (n=5) and 169.2 ± 39.8 nM (383.0 ± 33.2%) for α3β3γ2L (n=5). In contrast, the α1- and α5-containing receptors were much less sensitive to modulation by KRM-II-81, while the α2- and α3-containing receptors were robustly potentiated. From full concentration-response relationships, we found that the difference in sensitivity conferred by subunit subtype was largely in the relative potency of KRM-II-81, rather than its maximum efficacy (Figure 2B,2D). The average EC50 (and peak response) for potentiation of the response to GABA by KRM-II-81 was 1.73 ± 0.69 μM (316.9 ± 38.6%) for α1β3γ2L (n=5) compared to 101.9 ± 28.5 nM (350.1 ± 21.5%) for α2β3γ2L (n=5) and 60.9 ± 11.6 nM (352.2 ± 38.0%) for α3β3γ2L (n=5), which is
equivalent to a 17-fold and 28-fold selectivity on α2 and α3 subtypes over α1 subtype GABA<sub>A</sub> receptors, respectively. As a comparison, KRM-II-18B showed no significant selectivity among the three subtypes. Therefore, KRM-II-81 represents a novel and the first high-efficacy selective α2/α3 subtype-selective GABA<sub>A</sub> receptor PAM.

*Acetic acid- and lactic acid induced writhing*

Mice pre-treated with vehicle exhibited a mean of approximately 46 writhes following treatment of 0.6% acetic acid. As a positive control, the opioid morphine dose-dependently attenuated acetic acid-induced writhing \([F (3, 21) = 31.77, p < 0.05]\). *Post hoc* analyses indicated that the effects of 1.0 and 3.2 mg/kg of morphine were significantly different from vehicle and 0.32 mg/kg morphine. KRM-II-18B and KRM-II-81 both dose-dependently decreased the acetic acid-induced writhes \([KRM-II-18B: F (3, 22) = 11.16, p < 0.05, KRM-II-81: F (3, 23) = 7.05, p < 0.05]\). *Post hoc* analyses revealed that the effect of 3.2 and 10 mg/kg of KRM-II-18B were significantly different from those of vehicle and 1 mg/kg KRM-II-18B. The effects of 5.6 and 10 mg/kg of KRM-II-81 were significantly different from those of vehicle and 3.2 mg/kg KRM-II-81 (Figure 3). Larger doses of KRM-II-18B and KRM-II-81 were not studied because a pilot study found that a larger dose (32 mg/kg) produced marked sedation in mice, which may affect interpretation of the behavioral data.

When 0.32% lactic acid was used, mice pre-treated with vehicle exhibited approximately 27 writhes. Under this condition, morphine also dose-dependently reduced lactic-acid induced writhing \([F (3, 20) = 23.47, p < 0.05]\). *Post hoc* analyses indicated that the doses of 0.32 and 1.0 mg/kg of morphine significantly decreased the writhes as compared to vehicle and 0.1 mg/kg morphine treatment conditions. KRM-II-18B and KRM-II-81 both decreased the lactic acid-
induced writhes [KRM-II-18B: $F (3, 20) = 4.23, p < 0.05$, KRM-II-81: $F (3, 22) = 9.61, p < 0.05$]. Post hoc analysis revealed that 10 mg/kg KRM-II-18B significantly decreased the number of writhes as compared to vehicle- and 5.6 mg/kg-treated conditions; 10 mg/kg KRM-II-81 significantly decreased the number of writhes as compared to vehicle- and 3.2 mg/kg-treated conditions (Figure 3). In order to confirm the receptor mechanisms mediating the effects of the GABA$_A$ receptor PAMs, a dose of 3.2 mg/kg flumazenil was used as a pretreatment which showed near complete blockade of the antinociceptive effects of KRM-II-18B ($p < 0.05$, Figure 4A) and KRM-II-81 ($p < 0.05$, Figure 4B). This dose of flumazenil was chosen according to published literature, which showed significant blockade of the discriminative stimulus effects of benzodiazepines in rats (Gerak, 2011).

Acetic acid- and lactic acid induced nesting

When treated with vehicle, mice cleared 4.7 ± 0.2 out of 5 available zones by the end of the nesting period (data not shown). Acetic acid decreased the number of zones cleared to 1.4 ± 0.3. Morphine, KRM-II-18B, and KRM-II-81 all failed to significantly attenuate the decrease in cleared zones by acetic acid (Figure 5A). When treated with lactic acid (Figure 5B), mice again displayed a decrease in the number of zones cleared to 1.6 ± 0.3. Morphine increased the number of zones cleared [$F (3, 23) = 6.53, p < 0.05$]. Post hoc analyses revealed that morphine significantly increased the number of zones that were cleared at 3.2 mg/kg as compared to vehicle ($p < 0.05$). KRM-II-18B and KRM-II-81 also both dose-dependently increased the number of zones cleared [KRM-II-18B: $F(3, 26) = 5.97, p < 0.05$, KRM-II-81: $F (3, 27) = 3.55, p < 0.05$]. Post hoc analysis revealed that doses of 10 mg/kg of KRM-II-18B and 10 mg/kg of
KRM-II-81 significantly increased the number of zones cleared as compared to vehicle.

*Acid depressed locomotion*

When treated with vehicle, mice displayed a locomotor activity of 2323 ± 320.8 cm during the test period. Acetic acid (Figure 6A) and lactic acid (Figure 6B) decreased the locomotor activity to 577.1 ± 121.2 cm and 420.9 ± 62.44 cm, respectively, in agreement with previous reports (G. W. Stevenson et al., 2009). Acetaminophen pretreatment, at a dose that did not significantly change the locomotor activity in naïve mice, dose-dependently increased the locomotor activity of mice treated with acetic acid ($F$ (2, 16) = 3.39, $p < 0.05$) and lactic acid ($F$ (2, 17) = 11.30, $p < 0.05$). *Post hoc* analyses revealed that acetaminophen at a dose of 100 mg/kg produced a significant increase in locomotion as compared to vehicle- and 56 mg/kg-treatment conditions in both the acetic acid- and lactic acid-treated mice. However, pretreatment with KRM-II-18B and KRM-II-81 each failed to restore the locomotor activity of acetic acid- and lactic acid-treated mice. Larger doses of both PAMs were not studied because they produced other effects (e.g., altered locomotor activity, sedation) that are competing with behavioral measures of pain.

*Warm water tail withdrawal*

Under control conditions the average latencies for mice to remove their tails (mean ± SEM, in seconds) from 44 °C, 48 °C, and 52 °C water were 20 ± 0 (i.e., the maximal possible effect), 9.75 ± .87, and 3.42 ± .27, respectively. For the duration of the study, the baseline latencies did not significantly vary. Morphine dose-dependently increased the latency for mice to remove their tails from 48 °C water (Figure 7). *Post hoc* analysis revealed 10 mg/kg morphine significantly increased the tail withdrawal latency (18.6 ± 1.4) when compared to vehicle (9.6 ±
1.1). However, the α2/α3 subtype-selective GABA\textsubscript{A} PAM KRM-II-81 did not markedly change the tail withdrawal latency in 48 °C water.
Figure 1: Chemical structures of KRM-II-81 and KRM-II-18B

Figure 2: (A, B) Cells were transiently transfected with one of the α subtypes, as indicated, along with β3 and γ2L, and voltage clamped at -50 mV. Representative whole-cell currents are shown for 5 sec applications of GABA alone (gray) or GABA + 0.1 µM modulator (black). (C,
D) Concentration-response relationships for the positive allosteric modulators at α1-, α2, and α3-containing receptors. The peak current amplitude was divided by the response to GABA alone for each cell. Symbols (± SEM) show the average response from 5-7 cells. (E) Average enhancement of the current evoked to GABA by 0.1 µM (α4, α6) of the modulator indicated. The response was divided by the peak response to GABA alone for each cell. The dashed line at 100% indicates the response to GABA alone. Bars represent mean ± SEM (n=4-8).

Figure 3: Effects of morphine, KRM-II-18B, and KRM-II81 on A) 0.6% acetic acid and B) 0.32% lactic acid-induced writhing. All points represent the mean and error bars show SEM (n = 6–8 per group). Abscissa: dose of drug expressed as milligram per kilogram. Data point above “V” represents the number of writhes following administration of either A) acetic acid or B) lactic acid once pretreated with vehicle. Ordinate: number of writhes observed in the 25-minute observation period. Asterisks indicate data points that are significantly different from vehicle pretreatment (V) alone (*p < 0.05).
Figure 4: Effects of flumazenil on KRM-II-18B- and KRM-II-81-induced antinociception. Bars represent the mean and error bars show SEM. (n = 6–8 per group). Abscissa: pretreatment groups. Ordinate: total number of writhes induced by 0.32% lactic acid. A) KRM-II-18B pretreatment alone and with flumazenil. B) KRM-II-81 pretreatment alone and with flumazenil. Asterisks indicate significant difference in number of writhes in the presence and absence of flumazenil (*p < 0.05).
Figure 5: Effects of morphine, KRM-II-18B, and KRM-II-81 on A) acetic acid and B) lactic acid-depressed nesting (n=6-8). Data points show mean data and error bars show SEM. Abscissa: dose of drug expressed as milligram per kilogram. Data point above “V” (filled circle) represents the number of zones cleared following administration of either A) acetic acid or B) lactic acid.
once pretreated with vehicle. Ordinate: total number of zones cleared in the nesting procedure (60 min). Asterisks indicate that data points are significantly different from vehicle pretreatment (V) alone (*$p<$0.05).
**Figure 6:** Effects of acetaminophen (A, D), KRM-II-18B (B, E) and KRM-II-81 (C, F) on acetic acid (top) and lactic acid (bottom)-depressed locomotor activity. Data points show mean ± SEM. (n = 6–8 per group). Abscissa: dose of drug expressed as milligram per kilogram. Data point above “V” represents total distance traveled under control conditions (treated with vehicle). Data point above “A” represents total distance traveled following acid treatment. Ordinate: distance traveled in cm. Asterisks indicate data points significantly different from acetic acid (“A”) or lactic acid (“L”) alone (*p < 0.05).
**Figure 7:** The antinociceptive effects of morphine and KRM-II-81 in a warm water tail withdrawal assay in mice. Data are presented as mean ± SEM (n=6). Ordinate is latency (sec); Abscissa is the cumulative doses of the study drugs, in milligram per kilogram. Data above “V” represents the data collected after vehicle administration. Asterisks indicate data points significantly different from those tested under vehicle treatment (*p < 0.05).
This study examined the *in vitro* and *in vivo* effects of newly synthesized \( \text{GABA}_A \) receptor PAMs, KRM-II-18B and KRM-II-81. Cellular electrophysiological tracing identified KRM-II-81 as a \( \alpha_2/\alpha_3 \) subtype-selective PAM while KRM-II-18B was non-selective. Two compounds were then evaluated in mice models of pain-stimulated (writhing) and pain-suppressed (acid-depressed nesting and locomotion) behaviors. Additionally, the antinociceptive effects of KRM-II-81 was evaluated in a warm water tail withdrawal assay. Both PAMs significantly decreased acetic acid- and lactic acid-induced writhing, which was attenuated by the benzodiazepine receptor antagonist flumazenil. KRM-II-18B and KRM-II-81 restored lactic acid (but not acetic acid)-depressed nesting. In the assay of acid-depressed locomotion, acetaminophen but neither of the two \( \text{GABA}_A \) receptor PAMs was effective in attenuating the decrease in locomotion. In the warm water tail withdrawal assay, morphine dose-dependently increased the tail withdrawal latency, while the \( \alpha_2/\alpha_3 \) subtype-selective \( \text{GABA}_A \) PAM KRM-II-81 failed to produce antinociceptive effects. Combined, this study identified a novel high-efficacy \( \alpha_2/\alpha_3 \) subtype-selective PAM which showed significant antinociceptive effects in mouse assays of chemical stimulation-induced pain-like behaviors.

Given the critical role of spinal modulation of pain processing by \( \text{GABA}_A \)ergic neurons, one new strategy for novel analgesic discovery may involve the positive allosteric modulation of \( \alpha_2/\alpha_3 \) subtype-containing \( \text{GABA}_A \) receptors. Previous research has identified \( \text{GABA}_A \) receptor PAMs which has some selectivity on the individual \( \alpha \) subtypes and shows some promising results with specific antinociceptive effects. For example, NS11394 has functional efficacy
selectivity of $\alpha_5 > \alpha_3 > \alpha_2 > \alpha_1$ and produces significant antinociceptive effects in rat models of inflammatory and neuropathic pain but does not produce sedation at the same doses (Munro et al., 2008). However, given the highest efficacy of NS11394 at $\alpha_5$ subtype GABA$_A$ receptors, its side effect on cognitive impairment could be serious (Crestani & Rudolph, 2015). A more selective compound NS16085 demonstrated moderate to low efficacy at $\alpha_2$ and $\alpha_3$ subtypes, no efficacy at $\alpha_5$ subtypes and a slight negative efficacy at $\alpha_1$ subtypes, which was partially effective in the formalin test (de Lucas et al., 2015). In an effort to develop novel and high-efficacy selective $\alpha_2/\alpha_3$ subtype GABA$_A$ receptor PAMs, we discovered KRM-II-81. In this present study, we find KRM-II-81 is a selective $\alpha_2/\alpha_3$-selective GABA$_A$ receptor PAM in \textit{in vitro} characterization.

In order to examine the \textit{in vivo} activity of KRM-II-81, we used an acid-induced writhing test in mice to evaluate its potential antinociceptive effects. First, antinociceptive effects were assessed by observing the ability of KRM-I-81 to reduce acetic acid-induced writhing. Because both GABA$_A$ receptor PAMs only partially reduced 0.6% acetic acid-induced writhes, we hypothesized that such a chemical stimulus was too strong and these compounds might be more effective if a weaker painful stimulation were used. We then used 0.32% lactic acid-induced writhes to test the same doses of KRM-II-81 and KRM-II-18B. Under this condition, morphine and both KRM-II-81 and KRM-II-18B significantly decreased the number of acid-induced writhes. Pretreatment of the benzodiazepine-site antagonist flumazenil showed near complete blockade of the antinociceptive effects of KRM-II-81 and KRM-II-18B. This result confirmed that the antinociceptive effects of these GABA$_A$ receptor PAMs are primarily mediated through the benzodiazepine binding site of GABA$_A$ receptors.
Because pain not only stimulates nocifensive behaviors but also suppresses many adaptive behaviors, such as nesting or locomotion, measures of pain-depressed behaviors can provide new insights into the behavioral consequences of pain and the effects of candidate analgesics (Negus et al., 2015; Negus et al., 2006). We next examined the effects of the GABA\textsubscript{A} receptor PAMs on acid-depressed nesting behavior. While morphine, KRM-II-18B, and KRM-II-81 were effective in dose-dependently restoring nesting behavior in lactic acid-treated mice, no drug was effective in acetic acid-treated mice. When considered with the writhing data, these data support the notion that 0.6\% acetic acid may produce a greater noxious stimulus than 0.32\% lactic acid. In a previous study, morphine was partially effective in reversing nesting behavior in 0.32\% lactic acid-treated mice (Negus et al., 2015), and our results were consistent with that. More importantly, higher doses suppressed nesting behavior in non-acid treated (pain-free) mice (Negus et al., 2015). It is conceivable that given the higher strength of pain induced by 0.6\% acetic acid, morphine was able to reduce writhes but due to the nesting-suppressive effect of higher doses of morphine, the resultant effect was not statistically significant. Taken together, our results suggest that the ability of $\alpha_2/\alpha_3$ GABA\textsubscript{A} receptor PAMs to restore pain-depressed behavior may greatly depend on the degree of pain.

Pain also reduces the spontaneous locomotion in mice (Stevenson et al., 2009). Next, we examined whether the GABA\textsubscript{A} receptor PAMs could restore acid-depressed locomotion. Because acetaminophen is among the most commonly used medicines to relieve pain and morphine has well-documented effect to increase locomotion (Li, Shah, Patel, Rice, & France, 2013), which may complicate the interpretation of the data, we compared the novel PAMs with acetaminophen instead of morphine in this assay of pain-depressed behavior.
Acetaminophen pretreatment, at a dose that did not significantly change the locomotor activity in naïve mice, dose-dependently increased the locomotor activity of mice treated with acetic and lactic-acid. However, pretreatment with KRM-II-18B and KRM-II-81 each failed to restore the locomotor activity of acetic acid- and lactic acid-treated mice. The restoration of acid-depressed locomotion may require higher analgesic effectiveness than the alleviation of other pain-related behaviors since locomotion itself may intensify existing pain symptoms (Moseley et al., 2008; Stevenson et al., 2009; van Weering, Vollenbroek-Hutten, Kotte, & Hermens, 2007). That is, in order to restore locomotion, an analgesic would need to not only reduce the basal noxious stimulus, but also the additional pain resulting from movement. This could be the reason why both GABA_A receptor PAMs were not able to restore acid-depressed locomotion.

Although the nesting and locomotion assays used here were both assays of pain-depressed behavior, the effects of the PAMs differed, where the PAMs were effective in the nesting assay but not the locomotion assay. This finding suggests that the ability of the PAMs to alleviate pain-depressed behavior is dependent on the behavioral endpoint. This finding is consistent with previous reports. For example, the dose of analgesic required to restore acid-depressed locomotion was much higher than the dose required to restore acid-depressed feeding (Stevenson, Bilsky, & Negus, 2006; Stevenson et al., 2009). Other studies have demonstrated disparities in behavioral endpoints such as pain-induced saccharin preference versus acid-depressed locomotor activity (de la Puente et al., 2015) and pain-depressed wheel-running versus pain-depressed feeding (Miller, Picker, Schmidt, & Dykstra, 2011). Thus, the disparities in the findings of this study are not altogether surprising. Another somewhat surprising finding was that both PAMs showed similar effects in all the behavioral assays under
this condition despite the fact that KRM-II-81 was selective for α2/α3 subtypes while KRM-II-18B was a non-selective GABA_A receptor PAM. Benzodiazepines are non-selective GABA_A receptor PAMs and systemic drug administration typically does not show analgesic effects due to significant sedation. However, intrathecal benzodiazepine administration can produce analgesia (Tucker, Lai, Nadeson, & Goodchild, 2004; Tucker, Mezzatesta, Nadeson, & Goodchild, 2004). In this study, the doses of both PAMs used for pain studies did not significantly alter the spontaneous activity, suggesting minimal sedation. Yet both compounds produced clear antinociceptive actions. This could be due to the differential efficacy demands of the behavioral endpoints (i.e., antinociception has lower efficacy demand than sedation), or differential target engagement of both compounds in the central nervous system underlying the behaviors. More work needs to be done to decipher this interesting finding.

Next, we studied the ability of the novel α2/α3-selective GABA_A receptor PAM to produce antinociceptive effects in another nociception assay. This assay is primarily conducted to assess spinal reflexes to noxious thermal stimuli. Morphine has been shown to produce antinociceptive effects in the warm water tail withdrawal procedure (Bohn, Lefkowitz, & Caron, 2002; Thorn et al., 2011). Therefore, in the present study, we chose to use morphine as our positive control. As expected, morphine dose-dependently attenuated acute pain in the warm water tail withdrawal procedure. However, KRM-II-81 failed to produce antinociceptive effects, despite having produced antinociceptive effects in the acute model of visceral pain. This finding suggests that the ability of α2/α3-selective GABA_A receptor PAMs to attenuate acute pain may be dependent on the particular assay. Higher doses of KRM-II-81 altered locomotor activity. Therefore, higher doses of KRM-II-81 were not tested in the warm water tail withdrawal assay.

In conclusion, this study reported a novel α2/α3-selective GABA_A receptor PAM, which demonstrated good selectivity and significant antinociception without decreasing locomotion.
Importantly, because the efficacy of GABA<sub>A</sub> receptor PAMs at α5-subtype is closely related to cognitive impairment and compounds with α5-subtype efficacy such as NS11821 impairs memory and cognition (Zuiker et al., 2016) the lack of efficacy at α5 subtype GABA<sub>A</sub> receptors makes KRM-II-81 less likely to produce cognitive impairment. These results support the notion that developing subtype-selective GABA<sub>A</sub> receptor PAMs may be a viable strategy to discover novel analgesics. It should be noted that the GABA<sub>A</sub> receptor PAMs described here appear to be less effective than opioids for acid-induced visceral pain. This may not be surprising as acute pain condition may not involve marked spinal GABAergic disinhibition, a condition that GABA<sub>A</sub> receptor PAMs may be able to reverse. This is supported by studies using other GABA<sub>A</sub> PAMs. For example, the GABA<sub>A</sub> PAM NS11394 was only partially effective (33% reduction) in the formalin test (Munro et al., 2008). Future studies should examine the antinociceptive effects of KRM-II81 in more persisting chronic pain conditions such as nerve injury induced neuropathic pain.
CHAPTER 2B– ANALGESIA IN CHRONIC PAIN

INTRODUCTION

Chronic pain is one of the most common reasons for health-care visits. It affects more Americans than diabetes, heart disease, and cancer combined (AAPM, 2011). Opioids are commonly prescribed for chronic pain management; however, they have adverse effects that have become increasingly highlighted over the years. Such adverse effects include antinociceptive tolerance, respiratory depression, constipation, dependence, and abuse (Dahan, Aarts, & Smith, 2010; Inturrisi, 2002). Consequently, the development of novel effective analgesics, particularly for chronic pain, remains a clinical need.

Spinal GABA_A receptors have been shown to play a major role in pain processing (Knabl et al., 2008; Zeilhofer, Ralvenius, & Acuna, 2015). Previous studies have indicated that diminished glycinergic and GABAergic mediated-inhibition in the spinal cord, is a major contributing factor to inflammatory and neuropathic chronic pain (Di Lio et al., 2011; Munro et al., 2008). Thus, pharmacological restoration of GABAergic synaptic inhibition is a promising solution for pain management. As a result, GABA_A receptors have received increasing attention as pharmacological targets for pain control. KRM-II-81 and NS16085, have been shown to attenuate pain in acute pain models, visceral pain, and formalin-induced pain, respectively (de Lucas et al., 2015; Lewter et al., 2017). The objective of this study was to study the analgesic effects of α2/α3 subtype-selective GABA_A PAMs KRM-II-81 and NS16085 in rat models of inflammatory and neuropathic chronic pain.
CHAPTER 2B– ANALGESIA IN CHRONIC PAIN
MATERIALS AND METHODS

Subjects: Adult male Sprague-Dawley rats, (Envigo, Indianapolis, IN, USA) that were eight weeks old and weighed 200-250g upon arrival, were used in these studies. Rats were housed individually, and were on a 12/12 h light/dark cycle (all behavioral experiments were conducted during the light period. The animals had free access to water and food except during experimental sessions. Animals (n= 6 per group) were maintained and experiments were conducted in accordance with guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Institutional Animal Care and Use Committee, University at Buffalo, the State University of New York (Buffalo, NY) and with the 2011 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences, Washington, DC).

Induction of inflammatory and neuropathic pain: Inflammatory pain was induced by Complete Freund’s Adjuvant (CFA) inoculation in the rats, as previously described (Li, Thorn, Qiu, Peng, & Zhang, 2014). 0.1 mL of CFA, which consists of approximately 0.05 mg of Mycobacterium butyricum dissolved in paraffin oil, was injected into the right hind foot pad of anaesthetized rats (2 % isoflurane mixed with oxygen at a flow rate of 5/L min−1). The level of anesthesia was assessed by loss of righting reflex.

Neuropathic pain was induced by the chronic constriction injury (CCI) procedure, as previously described (Bennett & Xie, 1988). Rats were anaesthetized with a mixture that contained ketamine (60 mg/kg) and xylazine (5 mg/kg) i.p. prior to CCI surgery. Once the sciatic nerve was exposed, four ligatures (4-0 chromic gut; Roboz Surgical Instrument Co. Inc., Rockville,
MD) were placed around the sciatic nerve (around 1 mm apart). Ligatures were loosely placed around the nerve in order to assure that circulation through the epineural vasculature was not compromised.

**Mechanical hyperalgesia:** Mechanical hyperalgesia was measured two days after CFA inoculation, and 7 days after CCI surgery, using von Frey filaments (1.4 g – 26 g; North Coast Medical, Morgan Hill, CA). Rats were placed in elevated acrylic chambers with a mesh floor (IITC Life Science Inc., Woodland Hills, CA). von Frey filaments were applied perpendicularly to the medial plantar surface of the hind paw of the rat, until buckling of the filament occurred and was maintained for approximately 2 s. Starting with the lowest filament (1.4 g), von Frey filaments were applied in an ascending order until a behavioral response was elicited (withdrawal of the hind paw). Mechanical thresholds (paw withdrawal thresholds, PWT) correspond to the lowest force (g) that elicited the withdrawal of the hind paw in at least two of three applications. For the time course studies, rats received a single injection of KRM-II-81, NS16085, or vehicle immediately following the t= 0 min measurement, and were assessed every subsequent 15 min for 240 min. In experiments where the cumulative dosing procedure was used, measurements were recorded every 20 minutes, and immediately after each measurement, rats received the next dose of drug until the maximum threshold (26 g) was observed.

**Thermal hyperalgesia:** Thermal hyperalgesia was measured two days after CFA, and 7 days after CCI, using a plantar test apparatus (IITC Life Science Inc.), as described previously (Siemian, Obeng, Zhang, Zhang, & Li, 2016). Rats (n=6/group) were placed in acrylic elevated chambers with a glass base. The apparatus contains a heat source that radiated a light beam, under the glass base, to the hind paw. An adjustable angled mirror on the heat source allowed the
experimenter to apply the thermal stimulus to the correct area of the paw. The heat source was set with an active intensity of 40%, idle intensity of 10% and a cutoff time of 20 s. The paw withdrawal latency (PWL), the time from the start of the light beam, until the animal withdrew the paw from the heat stimulus was measured, as described previously (Hargreaves, Dubner, Brown, Flores, & Joris, 1988). Time-course effects of test drugs were studied, where rats received a single injection of either KRM-II-81, NS16085, or vehicle immediately following the t= 0 min measurement and were assessed every subsequent 30 min for 120 min. Measurements were taken in duplicate, approximately 1 minute apart and the average was used for statistical analysis.

**Data Analysis:** All graphs and statistical analyses were performed with the GraphPad Prism 7.0 program (GraphPad Software, San Diego, CA, USA). The mean values (± SEM) were calculated from individual animals for mechanical and thermal nociception assays, as well as PEAP. One-way or two-way repeated measurements ANOVA followed by post hoc Bonferroni’s test were used to determine the statistical significances. Critical values for the main or interaction effects were set at p<0.05. For the antagonist study, ED$_{50}$ values (± SEM) values were determined from the % MPE of each drug (Table 1). Effects were considered significant if 95% confidence limits (CL) values from tests with test compounds with antagonist or test compounds alone did not overlap. The pK$_{B}$ values were calculated using the following equation: pK$_{B}$=−log[B/(DR−1)]. In the equation, “B” is the dose of antagonist (flumazenil) in mol/kg and “DR” is the ED$_{50}$ of agonist combined with antagonist divided by the ED$_{50}$ of agonist alone.

**Drugs:** KRM-II-81 and NS16085 were obtained from Dr. James M. Cook (University of Wisconsin-Milwaukee) and synthesized according to published procedure (de Lucas et al., 2015,
Poe et al., 2016). KRM-II-81 was dissolved in a vehicle of 20% dimethyl sulfoxide (Amresco, Solon, OH) and 10% emulphor (Solvay, Cranbury, NJ), in 0.9% saline. NS16085 was dissolved in 5% Tween 80 in milliQ water. Flumazenil was purchased from Cayman Chemical (Ann Arbor, MI, USA) and was dissolved in a vehicle of 50% sterile water, 40% propylene glycol (Duda Diesel LLC, Madison, AL), and 10% ethanol.
CHAPTER 2B– ANALGESIA IN CHRONIC PAIN

RESULTS

Analgesic effects of α2/α3 GABA\textsubscript{A} PAMs were evaluated in a rat model of inflammatory pain. CFA inoculation into the right hindpaw of the rats produced mechanical and thermal hyperalgesia that persisted beyond the duration of the experiments, as described previously (David A. Thorn, Siemian, Zhang, & Li, 2015). Mean pre-CFA baseline values of (± S.E.M.) of 24.17 ± 0.99 g and 18.90 ± 0.60 seconds for paw withdrawal threshold (PWT) and paw withdrawal latency (PWL), were markedly reduced to average post-CFA values of 4.33 ± .18 g and 6.60 ± .38 seconds. To study the analgesic effects of α2/α3 GABA\textsubscript{A} PAMs KRM-II-81 and NS16085, we conducted time course effects of each drug in mechanical and thermal hyperalgesia assays, in separate groups of rats (Figure 8). Two-way ANOVA revealed significant dose × time interactions (F [54, 360] = 9.39, P< 0.05) and post hoc analyses found that 3.2 mg/kg KRM-II-81 significantly increased PWT from 45-135 min following injection, and 5.6 mg/kg KRM-II-81 significantly increased PWT 30-195 min following injection as compared with vehicle. NS16085 also significantly increased the PWT in CFA-treated rats. Two-way ANOVA revealed significant dose × time interactions (F [60, 375] = 7.57, P< 0.05) and post hoc analyses found that 5.6 mg/kg NS16085 significantly increased PWT 60-75 min following injection, 10 mg/kg NS16085 significantly increased PWT 30-90 min following injection, and 17.8 mg/kg NS16085 45-150 min following injection as compared with vehicle. Both GABA\textsubscript{A} PAMs failed to significantly increase PWL in the thermal nociception assay.
Analgesic effects of α2/α3 subtype-selective GABA\(_A\) PAMs were also evaluated in a rat model of neuropathic pain (Figure 9). CCI surgery produced mechanical and thermal hyperalgesia that persisted beyond the duration of the experiments. Mean pre-CCI baseline values of (± S.E.M.) of 22.79 ± 1.04 g and 16.76 ± 0.77 seconds for paw withdrawal threshold (PWT) and paw withdrawal latency PWL), were markedly reduced to average post-CCI values of 4.42 ± 0.17 g and 6.09 ± 0.31 second. KRM-II-81 significantly increased the PWT in CCI rats. Two-way ANOVA revealed significant dose × time interactions (F \([54, 360] = 6.54, P < 0.05\) and post hoc analyses found that 3.2 mg/kg KRM-II-81 significantly increased PWT from 45-75 min following injection, and 5.6 mg/kg KRM-II-81 significantly increased PWT 45-210 min following injection as compared with vehicle. NS16085 also significantly increased PWT in CCI rats. Two-way ANOVA revealed significant time x test interactions (F \([45, 300] = 6.17, P < 0.05\) and post hoc analyses found that 10 mg/kg NS16085 significantly increased PWT from 60-75 min following injection, and 17.8 mg/kg NS16085 significantly increased PWT from 15-135 min, following injection as compared to vehicle. Neither KRM-II-81 nor NS16085 demonstrated analgesic effects in the thermal nociception assay.

To verify the pharmacological action of KRM-II-81 and NS16085, we tested the effect of flumazenil (a benzodiazepine-site antagonist) pretreatment on the analgesic effects of KRM-II-81 and NS16085 (Figure 10). According to one-way repeated measures ANOVA, midazolam, KRM-II-81, and NS16085, all produced a significant main effect on PWT [F(3, 15) = 56.7 for midazolam, F(3, 23) = 1.87 for KRM-II-81, and [F(3, 23) = 16.8] in CFA-treated rats, using a multiple cycle cumulative dosing procedure (Figure 10). Post hoc analysis indicated that 3.2 and 5.6 mg/kg midazolam, 5.6 mg/kg KRM-II-81, and 10 and 17.8 mg/kg NS16085 produced
significant analgesic effects as compared to their respective vehicle controls. When administered prior to midazolam, 10 mg/kg flumazenil produced 2.27-fold rightward shifts of the midazolam dose-effect curve (pK_B = 4.53). Ten milligrams per kilogram of flumazenil produced a 2.73-fold rightward shift of the KRM-II-81 dose-effect curve (pK_B = 4.72), and 4.85-fold rightward shift of the NS16085 dose-effect curve (pK_B = 5.07). In contrast, 1.0 mg/kg flumazenil did not significantly shift dose-effect curves of GABA_A PAMs (see Table 1 for ED_{50} values).

According to one-way repeated measures ANOVA, midazolam, KRM-II-81, and NS16085, all produced a significant main effect on PWT [F(3, 15) = 19.8, for midazolam, F(3, 15) = 32.5, P < 0.05 for KRM-II-81, and F(3, 15) = 21.1, P < 0.05] in CCI rats, using a multiple cycle cumulative dosing procedure (Figure 2.10). Post hoc analysis indicated that 3.2 and 5.6 mg/kg midazolam, 3.2 and 5.6 mg/kg KRM-II-81, and 10 and 17.8 mg/kg NS16085 produced significant antinociceptive effects as compared to their respective vehicle controls. When administered prior to midazolam, 10 mg/kg flumazenil produced 2.71-fold rightward shifts of the midazolam dose-effect curve (pK_B = 4.7). 10 mg/kg flumazenil produced a 3.18-fold rightward shift of the KRM-II-81 dose-effect curve (pK_B = 4.93) and 3.22-fold rightward shift of the NS16085 dose-effect curve (pK_B = 4.83). In contrast, 1.0 mg/kg flumazenil did not significantly shift dose-effect curves of GABA_A PAMs (Table 1).
Figure 8: Analgesic effects of KRM-II-81 and NS16085 in CFA-treated rats (n=6/group).

Mechanical Nociception (A): Ordinates: paw withdrawal threshold (grams) measured by von Frey filaments; Abscissa, time (minutes) following injection of treatment drug. Filled-in symbols (gray) indicate time points that are significantly different from vehicle treatment condition. The dotted line indicates the mean paw withdrawal threshold of rats prior to receiving CFA. Thermal Nociception (B): Ordinates, paw withdrawal latency (seconds) measured by Hargreaves test; Abscissa, time (minutes) following injection of treatment of drug.
Figure 9: Analgesic effects of KRM-II-81 and NS16085 in CCI rats (n=6/group). Mechanical Nociception (A): Ordinates: paw withdrawal threshold (grams) measured by von Frey filaments; Abscissa, time (minutes) following injection of treatment drug. Filled-in symbols (gray) indicate time points that are significantly different from vehicle treatment condition ($p < 0.05$). The dotted line indicates the mean paw withdrawal threshold of rats prior to undergoing CCI surgery. Thermal Nociception (B): Ordinates, paw withdrawal latency (seconds) measured by Hargreaves test; Abscissa, time (minutes) following injection of treatment of drug.
Figure 10: Analgesic effects of GABA_A PAMs alone or in the presence of flumazenil pretreatment in A) CFA-treated rats and B) CCI-induced rats (n=6/group). Flumazenil attenuates midazolam-induced antinociception (left), KRM-II-81-induced antinociception (middle), and NS16085-induced antinociception (right), in the mechanical nociception assay. The dotted line indicates the mean paw withdrawal threshold of rats prior to receiving CFA or undergoing CCI surgery. Ordinates: paw withdrawal threshold (grams) measured by von Frey filaments; Abscissa, doses (mg/kg, i.p.) of midazolam (left), KRM-II-81 (middle), and NS16085 (right).
Table 1: ED$_{50}$ values (95% CL) of GABA$_A$ positive allosteric modulators alone or in combination with flumazenil pretreatment.

<table>
<thead>
<tr>
<th>Chronic Constriction Injury (CCI)</th>
<th>Positive Allosteric Modulator (PAM)</th>
<th>PAM + 1.0 mg/kg flumazenil</th>
<th>PAM + 10 mg/kg flumazenil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam</td>
<td>2.76 (2.15, 3.55)</td>
<td>3.01 (2.34, 3.88)</td>
<td>7.48 (5.64, 9.91)</td>
</tr>
<tr>
<td>KRM-II-81</td>
<td>2.83 (2.28, 3.51)</td>
<td>3.86 (3.27, 4.56)</td>
<td>9.00 (7.64, 10.6)</td>
</tr>
<tr>
<td>NS16085</td>
<td>9.07 (7.12, 10.5)</td>
<td>10.7 (7.94, 14.9)</td>
<td>29.2 (22.7, 37.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complete Freund's Adjuvant (CFA)</th>
<th>Positive Allosteric Modulator (PAM)</th>
<th>PAM + 1.0 mg/kg flumazenil</th>
<th>PAM + 10 mg/kg flumazenil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam</td>
<td>3.19 (2.79, 3.65)</td>
<td>3.83 (2.61, 5.63)</td>
<td>7.25 (5.86, 8.97)</td>
</tr>
<tr>
<td>KRM-II-81</td>
<td>3.92 (3.22, 4.78)</td>
<td>6.80 (5.70, 8.12)</td>
<td>10.7 (8.59, 13.2)</td>
</tr>
<tr>
<td>NS16085</td>
<td>8.57 (7.50, 9.80)</td>
<td>9.33 (8.03, 10.8)</td>
<td>41.6 (23.4, 74.1)</td>
</tr>
</tbody>
</table>
CHAPTER 2B– ANALGESIA IN CHRONIC PAIN

DISCUSSION

The primary findings of the current study were that α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs KRM-II-81 and NS16085 produced significant mechanical analgesia in rat models of inflammatory and neuropathic pain. Flumazenil pre-treatment attenuated the analgesic effects of KRM-II-81 and NS16085, confirming the role of the benzodiazepine-site in mediating analgesic activity of GABA<sub>A</sub> PAMs.

Previous studies have reported the antinociceptive effects of subtype-selective GABA<sub>A</sub> receptor PAMs in chronic pain models (Di Lio et al., 2011; Munro et al., 2008). However, very few of the GABA<sub>A</sub> PAMs that have been reported, are truly selective for α2/α3 subtype-containing GABA<sub>A</sub> receptors. GABA<sub>A</sub> PAMs such as HZ166, NS11394, L-838417, and TPA-023 show selectivity/efficacy at α5 subtypes (in addition to α2/α3) (Atack et al., 2006; Di Lio et al., 2011; R. M. McKernan et al., 2000; Mirza et al., 2008). Therefore, in this study we assessed the analgesic effects of two GABA<sub>A</sub> receptor PAMs, KRM-II-81 and NS16085, which were reported to be truly selective for α2/α3-subtypes (Lewter et al., 2017, de Lucas AG et al., 2015). In rat models of both inflammatory (Figure 8) and neuropathic pain (Figure 9), we found that both KRM-II-81 and NS16085 dose-dependently attenuated mechanical nociception in the von Frey assay. However, they failed to attenuate thermal nociception in the Hargreaves test. In order to confirm the validity of our thermal nociception assay, we studied the ability of morphine to significantly increase paw withdrawal latency in CFA-treated and CCI-induced rats. Morphine (3.2 and 10 mg/kg) significantly increased the paw withdrawal latency in both pain states (data
not shown). Thus, confirming the face validity of our method to measure thermal antinociception.

So far, many subtype-selective GABA<sub>A</sub> PAMs have been shown to attenuate both thermal and mechanical nociception (Di Lio et al., 2011; Nickolls et al., 2011). Therefore, it is unclear why differences between the two pain assays were observed in the present study. One possibility could be the lack of selectivity/efficacy at the α5-subtype. To date, the majority of studies assessing the analgesic effects of subtype-selective GABA<sub>A</sub> PAMs are performed using PAMs selective for α2/α3/α5 subtypes. Recently another PAM, MP-II-024, was reported to be selective for α2/α3-subtypes, however only its ability to reverse mechanical hyperalgesia was reported (Fischer et al., 2017). Further studies of the α5-subtype, and its role in pain processing, are necessary in order to support or oppose the notion of the α5-subtype mediating thermal nociception. Another possibility is the thermal stimulus was too strong for effects to be observed within the dose range that was used. In the case of KRM-II-81, higher doses (>10.0 mg/kg) were not tested, in that muscle-relaxant activity was observed at 17.8 mg/kg. In the case of NS16085 we tested up to 32 mg/kg and we still did not observe thermal hypersensitivity.

We have previously demonstrated that the benzodiazepine-site antagonist flumazenil, is able to attenuate the analgesic effect of KRM-II-81 in an acid-induced writhing procedure (Lewter et al., 2017). It is well known that GABA<sub>A</sub> PAMs mediate their effects through the benzodiazepine site receptor located between the α- and λ-subunit of the GABA<sub>A</sub> receptor (Costa, Guidotti, & Toffano, 1978; Möhler, 2011; Tallman, Thomas, & Gallager, 1978). In this present study, we sought to confirm the pharmacological actions of KRM-II-81, NS16085, and midazolam (Figure 10). As expected, we found analgesic effects of midazolam, KRM-II-81, and
NS16085 are attenuated with flumazenil pretreatment in both pain models. This is indicated by the significant rightward shifts of the dose-response curves, and the significant differences observed in ED50 values and 95% confidence intervals (Table 1). Effects were considered significant if 95% confidence limits (CL) values from tests with test compounds with antagonist or test compounds alone did not overlap. Together, these results confirm that analgesic effects of KRM-II-81 and NS16085 are mediated through the benzodiazepine-site of the GABA_A receptor.

In conclusion, previous studies of KRM-II-81 and NS16085 demonstrated the ability of these compounds to attenuate acute pain (de Lucas et al., 2015; Lewter et al., 2017). We previously reported KRM-II-81 to attenuate visceral pain in mice, while de Lucas et al., reported NS16085 to mediate analgesia after inflammatory injury induced by formalin. Up to this point, the ability of these PAMs (KRM-II-81 and NS16085) to be effective in models of chronic pain was unknown. This study demonstrated the ability of KRM-II-81 and NS16085 to produce analgesic effects in the von Frey assay. Additionally, the analgesic effects of both α2/α3 subtype-selective GABA_A PAMs are primarily mediated by the benzodiazepine-site of the GABA_A receptor. Collectively, these data support the notion that α2/α3-subtype selective GABA_A PAMs are effective for the management of chronic pain.
CHAPTER 2C- DEVELOPMENT OF ANALGESIC TOLERANCE

INTRODUCTION

For 50 years, extensive efforts have been made to develop new analgesics (Kissin, 2010). Yet, treating chronic pain still remains a clinical challenge. While opioids are commonly prescribed for treating chronic pain, there are various side-effects associated with their use. Some side-effects include constipation, physical dependence, and abuse (Dumas & Pollack, 2008; Ling, Mooney, & Hillhouse, 2011; Reis & Regunathan, 2000). Additionally, repeated treatment of opioids often leads to the development of analgesic tolerance. Tolerance is defined as a reduced effect (i.e. analgesia) following prolonged or repeated administration of a particular dose of drug (Dumas & Pollack, 2008). In the case of opioids, a decrease in analgesic efficacy may lead to patients consuming escalating doses in order to achieve consistent pain relief. This could ultimately lead to opioid addiction and overdose.

The development of tolerance, upon prolonged use, is not exclusive to opioids. Benzodiazepines are also limited by the development of tolerance to most of their pharmacological actions (Gravielle, 2016). Since, the majority of pharmacological effects of benzodiazepines are mediated through binding to the GABA_A receptor, changes in number, structure, and function of this receptor are thought to play a key role in tolerance development (Gravielle, 2016). Point-mutation and pharmacological studies have shown that different α-subtypes of the GABA_A receptor are associated with different pharmacological actions of benzodiazepines (Atack, 2010; Crestani et al., 2001; Crestani & Rudolph, 2015; McKernan et al., 2000; Rowlett, 2005). A recent mouse mutagenesis study revealed that analgesic tolerance
was developed from mice that only contained α3, α5, and α1-containing GABA_A receptors, while targeting only α2- subtype containing GABA_A receptors can produce analgesia without the development of analgesic tolerance (Ralvenius et al., 2015). Additionally, pharmacological studies have shown that positive allosteric modulators that are selective for α2, α3, and α5- containing GABA_A receptors do not develop analgesic tolerance (Di Lio et al., 2011). However, these α2/α3/α5 subtype-selective GABA_A PAMs are limited, due to their subtype-selectivity of the α5 subunit. The α5-subtype is associated with cognition and GABA_A PAMs selective for α5-subtypes could possibly impair cognition (Atack, 2010). Therefore, this study sought to assess the analgesic efficacy of two novel α2/α3 GABA_A receptor PAMs; KRM-II-81 and NS16085 before, during, and after repeated treatment, in rat models of inflammatory and neuropathic pain. The analgesic efficacy, following repeated treatment of KRM-II-81 and NS16085 were compared to repeated treatment of the nonselective GABA_A PAM, midazolam.
CHAPTER 2C- DEVELOPMENT OF ANALGESIC TOLERANCE
MATERIALS AND METHODS

Subjects: Described in Chapter 2B.

Induction of inflammatory pain: Described in Chapter 2B.

Induction of neuropathic pain: Described in Chapter 2B.

Mechanical hyperalgesia: Described in Chapter 2B.

Experimental Design: Starting two days after CFA treatment, and seven days after CCI surgery, mechanical hyperalgesia was measured on day 0 (before daily treatment), day 4 (during daily treatment), and day 8 or 12 (after treatment). On days 1-3, 5-7, and 9-11 (for KRM-II-81 and NS16085 groups), rats were treated with either saline or GABA_A PAMs [midazolam (5.6 mg/kg), KRM-II-81 (5.6 mg/kg), or NS16085 (10 mg/kg)]. Rats received injections of their assigned treatment twice a day (AM injections and PM injections) in their home cages. On days 0, 4, 8, and 12 only P.M. injections were given due to the mechanical nociception test done in the A.M.

Drugs: KRM-II-81 and NS16085 were provided by James M. Cook, PhD (University of Wisconsin-Milwaukee), and was dissolved as described in Chapter 2B: Midazolam (Akorn, Inc) was dissolved in saline. Doses were expressed as the weight of the drug in milligrams per kilogram of body weight and all drugs were administered intraperitoneally.

Data Analyses: The analgesic effects of midazolam, KRM-II-81, and NS16085 were quantified for each animal as percent possible effect (MPE) for each drug dose. The following equation was used to quantify % MPE: %MPE = [(Post-drug value for a behavioral response – Pre-drug value] / (Maximal possible effect – Pre-drug value) * 100.
for a behavioral response)/ (Pre manipulation [CFA or CCI] value – Pre-drug value for a behavioral response) × 100].

CHAPTER 2C- DEVELOPMENT OF ANALGESIC TOLERANCE

RESULTS

Under control conditions (CFA-naïve), rats withdrew their right hind paws when the force of the filament applied to the hind paw increased to 26g. CFA injection markedly decreased the PWT (e.g., midazolam group: 4.33 ± 0.33 g) in rats receiving CFA. Prior to receiving midazolam treatment (Day 0), midazolam dose-dependently increased the PWT, producing an ED$_{50}$ value (95% CLs) of 3.16 (2.76, 3.63) mg/kg (Figure 11). Daily treatment (twice a day) of midazolam resulted in progressively increased rightward shifts of the midazolam dose-effect curve observed on day 0. Three days of midazolam treatment increased the ED$_{50}$ value to 5.57 (4.56, 6.77) mg/kg, and produced a 1.76-fold rightward shift (observed on day 4). Seven days of midazolam treatment increased the ED$_{50}$ value to 9.16 (5.63, 14.9) mg/kg, and produced a 2.90-fold rightward shift (observed on day 8). Contrastingly, significant tolerance to the analgesic effects of midazolam [3.32 (2.96, 3.73)] mg/kg was not observed after 3 days [3.40 (2.87, 4.03) mg/kg] nor after 7 days [3.33 (3.09, 3.60) mg/kg] of daily saline treatment.

When rats with CFA-induced inflammatory pain were repeatedly treated with either of the α2/α3 subtype-selective GABA$_A$ PAMs, NS16085 (Figure 12, left) or KRM-II-81 (Figure 12, right), similar analgesic effects were observed day 0, day 4, and day 8 of the study (Table 2). Prior to receiving daily NS16085 treatment (Day 0), NS16085 dose-dependently increased the
PWT, producing an ED$_{50}$ value (95% CLs) of 11.4 (9.70, 13.5) mg/kg. Tolerance to the analgesic effects of NS16085 was not observed after 3 days [11.2 (10.2, 12.3) mg/kg] nor after 7 days [11.3 (9.45, 13.7) mg/kg] of daily NS16085 treatment. Therefore, rats were treated for 3 more days, and a cumulative dose-effect curve for NS16085 was reestablished on day 12. After 11 days of treatment, analgesic tolerance was not observed [11.6 (9.12, 14.8) mg/kg]. Prior to receiving daily KRM-II-81 treatment, KRM-II-81 dose-dependently increased the PWT, producing an ED$_{50}$ value (95% CLs) of 3.74 (3.45, 4.05) mg/kg. Tolerance to the analgesic effects of KRM-II-81 was not observed after 3 days [3.60 (2.81, 4.61) mg/kg], after 7 days [3.69 (3.09, 4.42) mg/kg], nor after 11 days [3.69 (2.84, 4.78) mg/kg] of daily KRM-II-81 treatment. Saline treatment failed to produce significant tolerance to the analgesic effects of KRM-II-81 and NS16085.

In the rats that received CCI surgery, CCI surgery markedly decreased the PWT (e.g., midazolam group: 4.67 ± 0.42 g in rats undergoing CCI). Prior to receiving midazolam treatment (Day 0), midazolam dose-dependently increased the PWT, producing an ED$_{50}$ value (95% CLs) of 3.02 (2.39, 3.83) mg/kg (Figure 13). Daily treatment (twice a day) of midazolam resulted in progressively increased rightward shifts of the midazolam dose-effect curve observed on day 0. Three days of midazolam treatment increased the ED$_{50}$ value to 4.28 (3.40, 5.39) mg/kg, and produced a 1.42-fold rightward shift. Seven days of midazolam treatment increased the ED$_{50}$ value to 8.05 (6.86, 9.45) mg/kg, and produced a 2.66-fold rightward shift. Contrastingly, significant tolerance to the analgesic effects of midazolam [2.89 (2.58, 3.24)] was not observed after 3 days [3.46 (2.89, 4.14)] mg/kg and after 7 days [2.96 (2.15, 4.10)] mg/kg of daily saline treatment. These data suggest that prolonged treatment with midazolam produced significant tolerance after 7 days.
When rats with CCI-induced neuropathic pain were repeatedly treated with either of the α2/α3 subtype-selective GABA_A PAMs, NS16085 (Figure 14, left) or KRM-II-81 (Figure 14, right), similar analgesic effects were observed day 0, day 4, and day 8 of the study (Table 2). Prior to receiving daily NS16085 treatment (Day 0), NS16085 dose-dependently increased the PWT, producing an ED_{50} value (95% CLs) of 11.0 (9.64, 12.6) mg/kg. Tolerance to the analgesic effects of NS16085 was not observed after 3 days [10.8 (8.75, 13.3) mg/kg], after 7 days [11.9 (8.43, 17.0) mg/kg] nor after 11 days [12.3 (9.51, 15.8) mg/kg] of treatment. Prior to receiving daily KRM-II-81 treatment, KRM-II-81 dose-dependently increased the PWT, producing an ED_{50} value (95% CLs) of 3.42 (2.81, 4.17) mg/kg. Tolerance to the analgesic effects of KRM-II-81 was not observed after 3 days [3.04 (2.88, 3.23) mg/kg], 7 days [3.58 (2.93, 4.36) mg/kg], nor after 11 days [3.34 (2.50, 3.34) mg/kg] of daily KRM-II-81 treatment. Saline treatment failed to produce significant tolerance to the analgesic effects of KRM-II-81 and NS16085.
Figure 11: Analgesic effects of midazolam before, during, and after 7 days of saline treatment (top) and midazolam treatment (bottom) in CFA-treated rats. Ordinate: percentage of maximal possible effect (100% MPE represents data from the baseline measure of the right hind paw before CFA treatment). Abscissa: dose of midazolam (mg/kg); n=6 per group.
Figure 12: Analgesic effects of NS16085 (left) and KRM-II-81 (right) before, during, and after 12 days of saline treatment (top) and GABA\textsubscript{A} PAMs (bottom) in CFA-treated rats. Ordinate: percentage of maximal possible effect. Abscissa: dose of GABA\textsubscript{A} PAMs (mg/kg); n=6 per group.
**Figure 13:** Analgesic effects of midazolam before, during, and after 7 days of saline treatment (top) and midazolam treatment (bottom) in CCI-induced rats. Ordinate: percentage of maximal possible effect. Abscissa: dose of midazolam (mg/kg); n=6 per group.
Figure 14: Analgesic effects of NS16085 (left) and KRM-II-81 (right) before, during, and after 12 days of saline treatment (top) and GABA_A PAMs (bottom) in CCI-induced rats. Ordinate: percentage of maximal possible effect. Abscissa: dose of GABA_A PAMs (mg/kg); n=6 per group.
**Table 2**: ED$_{50}$ values (95% CL) of GABA$_A$ positive allosteric modulators before (Day 0), during (Day 4), and after (Day 8/Day 12) daily treatment.

<table>
<thead>
<tr>
<th>Complete Freund's Adjuvant (CFA): ED$_{50}$ (95% CL) mg/kg values</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Day 0</td>
<td>Day 4</td>
<td>Day 8</td>
<td>Day 12</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3.16 (2.76, 3.63)</td>
<td>5.57 (4.56, 6.77)</td>
<td>9.16 (5.63, 14.9)</td>
<td>-</td>
</tr>
<tr>
<td>NS16085</td>
<td>11.4 (9.70, 13.5)</td>
<td>11.2 (10.2, 12.3)</td>
<td>11.3 (9.45, 13.7)</td>
<td>11.6 (9.12, 14.8)</td>
</tr>
<tr>
<td>KRM-II-81</td>
<td>3.74 (3.45, 4.05)</td>
<td>3.60 (2.81, 4.61)</td>
<td>3.69 (3.09, 4.42)</td>
<td>3.69 (2.84, 4.78)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chronic Constriction Injury (CCI): ED$_{50}$ (95% CL) mg/kg values</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Day 0</td>
<td>Day 4</td>
<td>Day 8</td>
<td>Day 12</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3.02 (2.39, 3.83)</td>
<td>4.28 (3.40, 5.39)</td>
<td>8.05 (6.86, 9.45)</td>
<td>-</td>
</tr>
<tr>
<td>NS16085</td>
<td>11.03 (9.64, 12.6)</td>
<td>10.8 (8.75, 13.3)</td>
<td>11.9 (8.43, 17.0)</td>
<td>12.3 (9.51, 15.8)</td>
</tr>
<tr>
<td>KRM-II-81</td>
<td>3.42 (2.81, 4.17)</td>
<td>3.04 (2.88, 3.23)</td>
<td>3.58 (2.93, 4.36)</td>
<td>3.34 (2.50, 3.34)</td>
</tr>
</tbody>
</table>
CHAPTER 2C- DEVELOPMENT OF ANALGESIC TOLERANCE

DISCUSSION

The primary findings of the current study were that prolonged treatment of α2/α3 subtype-selective GABA_A PAMs, KRM-II-81 and NS16085, at doses that produced near maximal analgesia, did not develop significant tolerance in two rat models of chronic pain. However, prolonged treatment of the nonselective midazolam, at a dose that produced near maximal analgesia, developed significant tolerance in both inflammatory and neuropathic pain states.

While benzodiazepines exert many desirable effects, their use is associated with many side effects precluding their long-term use, including the rapid development of tolerance. Once tolerance is developed, dose escalation is required in order to maintain adequate effects associated with benzodiazepines. In the present study, twice-daily treatment of midazolam (5.6 mg/kg) resulted in the rapid development of analgesic tolerance, after 3 consecutive days in the inflammatory pain model, and after 7 consecutive days in the neuropathic pain model. These results are consistent with other studies that have demonstrated loss of benzodiazepine activity due to prolonged treatment. For example, tolerance to the antinociceptive effects of diazepam developed after 10 days of diazepam treatment (1 mg kg-1/day) in rats, using the tail-flick test (Zambotti et al., 1991).

Our understanding of how benzodiazepines tend to lose their efficacy over time (i.e., tolerance) has not been well understood (Vinkers & Olivier, 2012). Nevertheless, a recent study using triple GABA_A receptor point-mutated mice demonstrated that targeting only α2-containing GABA_A receptors achieves robust analgesia devoid of the development of analgesic
tolerance (Ralvenius et al., 2015). In the present study, twice-daily treatment of α2/α3 subtype-selective GABA\(_A\) receptor PAMs KRM-II-81 (5.6 mg/kg) and NS16085 (10 mg/kg) failed to produce tolerance to mechanical analgesia, after 11 consecutive days of treatment in both inflammatory and neuropathic pain models. These findings are consistent with a study done by Di Lio et al., where α1-sparing GABA\(_A\) receptor PAM - HZ166 showed no loss of analgesic activity after a 9-day chronic treatment period in CCI-induced mice (Di Lio et al., 2011). Additionally, α1-sparing benzodiazepine-site ligand L-838, 417 is effective against inflammatory and neuropathic pain and devoid of analgesic tolerance upon prolonged treatment (Knabl et al., 2008). Despite, HZ166 and L-838, 417 possessing α5-GABA\(_A\) specificity (in addition to α2/α3 subtypes), both compounds still failed to produce significant tolerance upon repeated use. This suggests that improved α2-GABA\(_A\) specificity may not be the sole contributor to the lack of tolerance observed in these particular studies. Subtype-selective GABA\(_A\) PAMs L-838, 417, HZ166, KRM-II-81 and NS16085 all have little to no pharmacological activity at α1-subtype containing GABA\(_A\) receptors. Thus, the lack of α1-GABA\(_A\) specificity may greatly influence the lack of analgesic tolerance observed in previous studies (HZ166 and L-838, 417) and in the present study (KRM-II-81 and NS16085).

In conclusion, α2/α3 subtype-selective GABA\(_A\) PAMs are able to produce significant mechanical analgesia devoid of tolerance development up to 11-consecutive days, in models of inflammatory and neuropathic pain. Contrastingly, prolonged treatment of the nonselective midazolam produced significant tolerance after just 3 days in the inflammatory pain model, and after 7 days in the neuropathic pain model. Taken together, findings from this study further reinforce the notion of α2/α3 subtype-selective GABA\(_A\) positive allosteric modulators as a novel class of analgesics.
Combination therapy, combining more than one drug in a treatment regimen, has been used in various clinical areas such as asthma, oncology, and hypertension (Juniper et al., 2002; Law et al., 2003; Lilenbaum, Langenberg, & Dickersin, 1998). The aim in using combination therapy is to achieve similar or better analgesic effects, while simultaneously reducing undesired side effects that are likely to result from higher doses of a single drug (Gilron et al., 2013).

While monotherapies are effective under some conditions, there are some cases by which combination therapy has been shown to be more effective. For example, combining blood pressure-lowering drugs from different classes was shown to be approximately 5 times more effective than doubling the dose of 1 drug (Wald, Law, Morris, Bestwick, & Wald, 2009).

More recently, combination therapy has been used to manage pain. More than half of chronic pain patients are receiving more than one analgesic concurrently (Berger et al., 2012). Even with recent efforts, combination pharmacotherapy for non-cancer pain management remains an important and understudied strategy. Despite a relatively small number of clinical studies comparing the effectiveness of combination drug therapy with monotherapy for pain management, some positive trials have emerged. For example, both preclinical and clinical studies have shown combinations of gabapentin and opioids (morphine or oxycodone) to enhance the antinociceptive effects of either drug alone (Gilron et al., 2005; Vorobeychik, Gordin, Mao, & Chen, 2011). While the potential use of subtype-selective GABA<sub>A</sub> positive allosteric modulators as analgesics has been explored, the potential use of these compounds in combination therapy has yet to be investigated. Thus, the objective of the present study was to
examine the interactions between GABA<sub>A</sub> receptor PAMs (midazolam, and α2/α3 subtype-selective PAMs; KRM-II-81 and NS16085) and commonly prescribed opioids (morphine and oxycodone) using a rat model of inflammatory pain.
CHAPTER 3- COMBINATION THERAPY

MATERIALS AND METHODS

Subjects: Described in Chapter 2B.

Induction of inflammatory pain: Described in Chapter 2B.

Mechanical hyperalgesia: Described in Chapter 2B.

Schedule-controlled responding: The food maintained operant responding experiments were conducted in chambers that were enclosed in sound-attenuating, ventilated enclosures (Coulbourn Instruments Inc. Allentown, PA). Food-maintained operant responding experiments were conducted in commercially available chambers located within sound-attenuating, ventilated enclosures (Coulbourn Instruments Inc., Allentown, PA), as previously described (An, Zhang, Winter, & Li, 2012). Operant chambers contained two levers; responses on both the active lever (left) and the inactive (right) lever were recorded. There were no programmed consequences for responses on the inactive lever. Data were collected using Graphic State 3.03 software and an interface (Coulbourn Instruments, Inc.). Two groups of rats (n =8 per group) were trained to press a lever for a food pellet under a multiple-cycle procedure (FR10). Each cycle of the six cycle program begins with a 15 min inactive period, during which the chamber was dark and responses had no programmed consequence, followed by a 5 min response period, during which a light above the active lever was illuminated and rats could receive food pellets (45 mg dustless precision pellets; Bio Serv Inc, Frenchtown, NJ) for responses on the active lever. The light was terminated after the delivery of five food pellets or after the 5 min response period had elapsed, whichever occurred first. Daily sessions consisted of six cycles, and the response rates averaged across all six cycles within a session was required not to vary by more than 20% for 2 days prior to each test, as previously described (An et al., 2012).
During test sessions, rats received drugs in combination at the beginning of each inactive period in a cumulatively dosed manner.

**Data Analyses:** Analgesic effects of the drugs and drug combinations studied were quantified for each animal as %MPE for each dose. The formula for %MPE is described in chapter 2. To construct analgesic dose-effect curves, %MPEs were averaged within each group (±SEM) and plotted as a function of dose. Rate of schedule-controlled responding is expressed as a percentage of the vehicle control response rate. For each cycle of a test session, the control response rate for an individual rat was the average response rate of the corresponding cycle from three vehicle sessions immediately prior to the test. These percentages were averaged across eight rats (± SEM) and plotted as a function of dose. Log (ED$_{50}$) (± 95% confidence limits [CLs]) values were determined from %MPE for each animal within a particular group and averaged within the group to calculate the ED$_{50}$ values for each drug.

For the study that examined the interactions between GABA$_A$ PAMs (midazolam, KRM-II-81, and NS16085) and Opioids (oxycodone and morphine), a fixed ratio dose-addition analysis method was used as previously described (An et al., 2012; J. X. Li, Crocker, Koek, Rice, & France, 2011; J. X. Li, Zhang, & Winter, 2011). Briefly, two drugs were combined in fixed ratios (1:3, 1:1, and 3:1) and administered as one dose of combinations per test. The doses of the individual drugs were determined based on the relative potency (determined by ED$_{50}$ values) of the drugs and the fixed rations that were used. For example, the 1:1 ratio of oxycodone/KRM-II-81 consisted of $1 \times$ ED$_{50}$ of oxycodone (1.13 mg/kg) and $1 \times$ ED$_{50}$ of KRM-II-81 (3.84 mg/kg) from the mechanical nociception test. Fractions of this mixture (the combined 0.125 ×, 0.25 ×, 0.5 ×, 1 × ED$_{50}$ values of oxycodone and KRM-II-81) were administered consecutively by a cumulative dosing procedure to complete one dose-effect curve test with 20 min interval times.
Using this method, the 1:3 ratio consisted of $0.5 \times \text{ED}_{50}$ value of oxycodone and $1.5 \times \text{ED}_{50}$ value of KRM-II-81; the 3:1 ratio consisted of $1.5 \times \text{ED}_{50}$ value of oxycodone and $0.5 \times \text{ED}_{50}$ value of KRM-II-81. In the instance that the \text{ED}_{50} value of a drug could not be calculated (due to low efficacy) the \text{ED}_{50} value for another behavioral assay was used. For example, the \text{ED}_{50} value of KRM-II-81 was not calculated in the thermal nociception assay, therefore the \text{ED}_{50} value of KRM-II-81 from the mechanical nociception assay was used. Isobolograms were constructed to visually represent the nature of the drug interactions as additive, supra-additive, or infra-additive.

Dose-addition analysis was also performed as previously described (Tallarida, 2001). When both drugs were active in an assay, expected additive \text{ED}_{50} values ($\pm 95\% \text{ CL}$) ($Z_{\text{add}}$) were calculated from the equation $Z_{\text{add}} = fA + (1 - f)B$, where $A$ is the \text{ED}_{50} of 2-BFI alone, $B$ is the \text{ED}_{50} of the opioid alone, and $f$ is the fractional multiplier of $A$ in the computation of the additive total dose (e.g., $f = 0.5$ when fixed ratio was 1:1). Experimental \text{ED}_{50} values ($\pm 95\% \text{ CL}$) ($Z_{\text{mix}}$) were determined from the 1:3, 1:1, and 3:1 combinations and were defined as the sum of the \text{ED}_{50} values of both drugs in the combination. Effects were considered significant if the $Z_{\text{add}}$ and $Z_{\text{mix}}$ 95% confidence limits did not overlap. If $Z_{\text{mix}}$ was significantly less than $Z_{\text{add}}$, the interaction was considered supra-additive. If $Z_{\text{mix}}$ was significantly greater than $Z_{\text{add}}$, the interaction was considered infra-additive.

**Drugs:** Oxycodone hydrochloride and morphine sulfate was provided Research Technology Branch, National Institute on Drug Abuse, National Institutes of Health (Rockville, MD, USA) and dissolved in physiological saline. KRM-II-81 and NS16085 were synthesized (as described in Chapter 2). Doses are expressed as mg per kg body weight.
CHAPTER 3- COMBINATION THERAPY

RESULTS

Under control conditions, rats displayed a mean paw withdrawal threshold (PWT) of 23.4 ± 0.87g for the von Frey test and a mean paw withdrawal latency (PWL) of 18.0 ± 0.49 seconds for the Hargreaves test. Following CFA injection into the right hindpaw, PWT and PWL values reduced to average post-CFA values of 4.67 ± 0.20g and 8.17 ± 0.42 seconds. For each group of rats (n=6 per group), the drug or drug combination used in the von Frey test was the same used in the Hargreaves test.

To demonstrate the analgesic effects of oxycodone, morphine, midazolam, KRM-II-81, and NS16085, cumulative dose tests of each drug alone were performed (Figure 15). Oxycodone dose-dependently increased the PWT and PWL (one-way ANOVA: $F[4, 25] = 70.4$, $p <0.05$ for PWT, $F[4, 25] = 32.6$, $p <0.05$ for PWL). Post hoc analyses indicated significant effects at 1.0-3.2mg/kg oxycodone for PWT and 0.32-3.2 mg/kg oxycodone for PWL. Morphine dose-dependently increased the PWT and PWL (one-way ANOVA: $F[3, 20] = 144$, $p <0.05$ for PWT, $F[3, 20] = 53.3$, $p <0.05$ for PWL). Post hoc analyses indicated significant effects at 3.2-10mg/kg morphine for both PWT and PWL. The nonselective benzodiazepine midazolam dose-dependently increased the PWT and PWL (one-way ANOVA: $F[3, 20] = 150$, $p <0.05$ for PWT, $F[4, 25] = 71.5$, $p <0.05$ for PWL). Post hoc analyses indicated significant effects at 3.2-5.6 mg/kg midazolam for PWT and 1.78-5.6 mg/kg midazolam for PWL. The α2/α3 subtype-selective GABA$_A$ receptor PAMs KRM-II-81 and NS16085 both dose-dependently increased the PWT in the von Frey test, (one-way ANOVA: $F[4, 25] = 60.6$, $p <0.05$ for KRM-II-81, $F[4, 25] = 29.9$, $p <0.05$ for NS16085), but not the Hargreaves test. Post hoc analyses indicated significant effects at 3.2-5.6 mg/kg KRM-II-81 and 10-32 mg/kg NS16085.
NS16085 for PWT. The dose-effect curves of all the drugs mentioned above are presented as %MPE in Figure 15. The ED50 values (95% CLs) of the drugs alone for the von Frey and Hargreaves tests are presented in Table 3. The rank order of potency was oxycodone > midazolam > morphine > KRM-II-81 > NS16085 in both assays. The calculated potency ratio between midazolam and oxycodone was 2.70 for the von Frey assay and 2.92 for the Hargreaves assay. The calculated potency ratio between KRM-II-81 and oxycodone was 3.5 for the von Frey assay. The calculated potency ratio between NS16085 and oxycodone was 8.52 for the von Frey assay. Since KRM-II-81 and NS16085 failed to dose-dependently increase PWL in the Hargreaves test, the ED50 values from the von Frey test was used to calculate the potency ratio between oxycodone and KRM-II-81 (4.22) and oxycodone and NS16085 (10.6) for the Hargreaves test. In the following combination studies, these potency ratios were used to calculate the respective doses of each drug in combination. For example, under a fixed ratio of 1:1, every 1 mg/kg of oxycodone that was administered in the von Frey assay was accompanied by 2.70 mg/kg of midazolam. The combined 1/8×, 1/4×, 1/2×, and 1×ED50s of oxycodone and GABA\textsubscript{A} PAMs were administered consecutively by cumulative dosing procedure to complete one dose-effect curve test.

Using this approach, the analgesic effects of oxycodone in combination with GABA\textsubscript{A} PAMs: midazolam, KRM-II-81, and NS16085 were measured in the von Frey assay (Figure 16). All fixed ratio combinations of oxycodone and midazolam dose-dependently increased the PWT (one-way ANOVA: $F [4, 20] = 17.8$, $p < 0.05$ for 1:3, $F [4, 20] = 59.9$, $p < 0.05$ for 1:1, $F [4, 24] = 30.6$, $p < 0.05$ for 3:1. Post hoc analyses indicated significant effects at 2.29-4.58 mg/kg midazolam for 1:3, 1.5-3.05 mg/kg midazolam for 1:1, 0.76-1.5 mg/kg midazolam for 3:1. All fixed ratio combinations of oxycodone and KRM-II-81 dose-
dependently increased the PWT (one-way ANOVA: $F_{[4, 25]} = 65.1$, $p < 0.05$ for 1:3, $F_{[4, 25]} = 36.8$, $p < 0.05$ for 1:1, $F_{[4, 25]} = 22.0$, $p < 0.05$ for 3:1. Post hoc analyses indicated significant effects at 2.88-5.76 mg/kg KRM-II-81 for 1:3, 1.92-3.84 mg/kg KRM-II-81 for 1:1, 0.96-1.92 mg/kg KRM-II-81 for 3:1. All fixed ratio combinations of oxycodone and NS16085 dose-dependently increased the PWT (one-way ANOVA: $F_{[4, 25]} = 13.5$, $p < 0.05$ for 1:3, $F_{[5, 30]} = 21.8$, $p < 0.05$ for 1:1, $F_{[4, 25]} = 30.6$, $p < 0.05$ for 3:1. Post hoc analyses indicated significant effects at 7.22-14.4 mg/kg NS16085 for 1:3, 4.82-19.3 mg/kg NS16085 for 1:1, 2.41-4.82 mg/kg NS16085 for 3:1. The ED50 values (95 CLs) of oxycodone and GABA$_A$ PAMs in the Hargreaves test are listed in Table 2. In order to rule out the notion that behavioral suppression (e.g., motor impairment) may have influenced the analgesia observed in the two nociceptive assays, response rate-suppressing effects were also studied and plotted for comparison (Figure 16). The same fixed ratio dose combinations that were used for the von Frey assay were used to test food-maintained operant responding in pain-free animals. Under all fixed ratios of oxycodone and $\alpha2/\alpha3$ subtype-selective GABA$_A$ PAMs (KRM-II-81 and NS16085), significant suppression was not observed ($p > 0.05$). There was a noticeable decrease in response-rates under two of the fixed ratios of oxycodone and the nonselective GABA$_A$ PAM midazolam. Under the 1:1 and 3:1 oxycodone and midazolam combinations, significant suppression was observed with the ANOVA analysis ($p = 0.04$ for 1:1, $p = 0.001$ for 3:1), but not the post hoc analysis ($p = 0.90$ at 3.05 mg/kg midazolam for 1:1, $p = 0.07$ at 1.5 mg/kg midazolam for 3:1).

Isobolographic analyses indicated that the ED50 ($\pm$ 95% CLs) values of the oxycodone-midazolam mixtures in the 1:1 ratio fell within the confidence limits of the line of additivity in the von Frey assay, suggesting simple additive interactions in these combinations (Figure 17).
1:3 and 3:1 ratios fell below the line of additivity, which indicates supra-additive (synergistic) interactions. In the Hargreaves assay, all fixed ratios fell within the confidence limits of the line of additivity. ED50 (± 95% CLs) values of the oxycodone-NS16085 mixtures all fell within the confidence limit intervals of the line of additivity in the von Frey test. In the Hargreaves test, NS16085 alone was not effective. Thus, there is a modified line of additivity (plotting the oxycodone ED50 value) plotted in the isobolograms. All ED50 (± 95% CLs) values fell to the left of the plotted oxycodone ED50 value. The ED50 (± 95% CLs) values of the oxycodone-KRM- II-81 mixtures all fell within the confidence limit intervals of the line of additivity in the von Frey test, and to the left of the oxycodone ED50 value in the Hargreaves test. Dose addition analysis confirmed these results (Table 3).

Next, we sought to study the interactions of GABA<sub>A</sub> PAMs and another commonly prescribed opioid, morphine. The analgesic effects of morphine in combination with GABA<sub>A</sub> PAMs: midazolam, KRM-II-81, and NS16085 were measured in the von Frey assay (Figure 18). All fixed ratio combinations of morphine and midazolam dose-dependently increased the PWT (one-way ANOVA: \( F[4, 25] = 30.4, p <0.05 \) for 1:3, \( F[4, 25] = 18.2, p <0.05 \) for 1:1, \( F[4, 25] = 21.3, p <0.05 \) for 3:1. Post hoc analyses indicated significant effects at 2.29-4.58 mg/kg midazolam for 1:3, 1.5-3.05 mg/kg midazolam for 1:1, 0.76-1.5 mg/kg midazolam for 3:1. All fixed ratio combinations of morphine and KRM-II-81 dose-dependently increased the PWT (one-way ANOVA: \( F[4, 25] = 49.1, p <0.05 \) for 1:3, \( F[4, 25] = 49.0, p <0.05 \) for 1:1, \( F[4, 25] = 178, p <0.05 \) for 3:1. Post hoc analyses indicated significant effects at 2.88-5.76 mg/kg KRM-II-81 for 1:3, 1.92-3.84 mg/kg KRM-II-81 for 1:1, 0.96-1.92 mg/kg KRM-II-81 for 3:1. All fixed ratio combinations of morphine and NS16085 dose-dependently increased the PWT (one-way ANOVA: \( F[4, 25] = 47.8, p <0.05 \) for 1:3, \( F[4, 25] = 47.8, p <0.05 \) for 1:1, \( F[4, 25] = 49.0, p <0.05 \) for 3:1. Post hoc analyses indicated significant effects at 2.88-5.76 mg/kg KRM-II-81 for 1:3, 1.92-3.84 mg/kg KRM-II-81 for 1:1, 0.96-1.92 mg/kg KRM-II-81 for 3:1.
[4, 20] = 24.1, p <0.05 for 1:1, F [5, 30] = 65.5, p <0.05 for 3:1. Post hoc analyses indicated significant effects at 7.22-14.4 mg/kg NS16085 for 1:3, 4.82-9.63 mg/kg NS16085 for 1:1, 2.41-9.64 mg/kg NS16085 for 3:1. The ED50 values (95 CLs) of morphine and GABA<sub>A</sub> PAMs in the Hargreaves test are listed in Table 2. Response rate-suppressing effects were also studied and plotted for comparison (Figure 18). The same fixed ratio dose combinations that were used for the von Frey assay were used to test food-maintained operant responding in pain-free animals. Under all fixed ratios of morphine-KRM-II-81 and morphine-NS16085 interactions, significant suppression was not observed (p > 0.05). However, under all fixed ratios of morphine and midazolam, significant suppression was observed (one-way ANOVA: F [4, 26] = 8.48, p <0.05 for 1:3, F [4, 23] = 11.0, p <0.05 for 1:1, F [4, 28] = 14.0, p <0.05 for 3:1. Post hoc analyses indicated significant effects at 2.29-4.58 mg/kg midazolam for 1:3, 3.05 mg/kg midazolam for 1:1, 1.52 mg/kg midazolam for 3:1.

Isobolographic analyses indicated that the ED50 (± 95% CLs) values of the morphine-midazolam mixtures in the 1:1 fell above the line of additivity, indicating infra-additivity, values in the 3:1 ratios fell within the confidence limits of the line of additivity, and 1:3 fell below the line of additivity in the von Frey assay (Figure 19). In the Hargreaves assay, ED50 (± 95% CLs) values in the 1:3 ratio fell within the line of additivity. ED50 (± 95% CLs) values from the 1:1 and 3:1 dose ratios fell below the confidence limits of the line of additivity. ED50 (± 95% CLs) values of the morphine-NS16085 mixtures all fell within the confidence limit intervals of the line of additivity in the von Frey test. In the Hargreaves test, all ED50 (± 95% CLs) values fell to the left of the plotted morphine ED50 value. The ED50 (± 95% CLs) values of the morphine-KRM-II-81 mixtures from the 3:1 dose ratio fell within the confidence limit intervals of the line of additivity in the von Frey test, and values from the 1:3 and 1:1 ratios fell below the line of
additivity. In the Hargreaves test, all dose ratios fell to the left of the morphine ED50 value. Dose addition analysis confirmed these results (Table 3).
**Figure 15:** Percent maximal possible effects of oxycodone, morphine, and GABA_A receptor positive allosteric modulators on CFA-treated rats. Ordinates: percentage of maximal possible effect in von Frey (top) and Hargreaves (bottom) assay; Abscissa, drug dose (mg/kg).
Table 3: ED50 values (95%CL) for individual drugs in the von Frey test and Hargreaves' test. For drug combinations, only the ED50 values of the GABA_A PAMs are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>von Frey</th>
<th>Hargreaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>3.17 (2.59, 3.89)</td>
<td>2.99 (2.45, 3.65)</td>
</tr>
<tr>
<td>Midazolam</td>
<td>3.05 (2.96, 3.73)</td>
<td>2.66 (1.95, 2.67)</td>
</tr>
<tr>
<td>KRM-II-81</td>
<td>3.84 (3.13, 4.72)</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>NS16085</td>
<td>9.63 (7.39, 12.5)</td>
<td>&gt; 17.8</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>1.13 (0.90, 1.43)</td>
<td>0.91 (0.56, 1.47)</td>
</tr>
<tr>
<td>Oxycodone/Midazolam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>1.96 (1.34, 2.87)</td>
<td>1.08 (0.41, 2.90)</td>
</tr>
<tr>
<td>1:1</td>
<td>1.03 (0.83, 1.30)</td>
<td>0.68 (0.35, 1.34)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.76 (0.58, 1.00)</td>
<td>0.52 (0.37, 0.73)</td>
</tr>
<tr>
<td>Oxycodone/KRM-II-81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>3.29 (2.77, 3.91)</td>
<td>3.55 (1.98, 6.36)</td>
</tr>
<tr>
<td>1:1</td>
<td>1.76 (1.32, 2.32)</td>
<td>2.80 (1.54, 5.09)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.93 (0.68, 1.27)</td>
<td>0.67 (0.32, 1.40)</td>
</tr>
<tr>
<td>Oxycodone/NS16085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>6.36 (4.02, 10.1)</td>
<td>11.0 (7.07, 17.2)</td>
</tr>
<tr>
<td>1:1</td>
<td>4.60 (2.91, 7.29)</td>
<td>3.34 (1.99, 5.62)</td>
</tr>
<tr>
<td>3:1</td>
<td>2.32 (1.81, 2.96)</td>
<td>1.16 (0.57, 2.37)</td>
</tr>
<tr>
<td>Morphine/Midazolam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>1.84 (1.35, 2.51)</td>
<td>1.93 (1.28, 2.91)</td>
</tr>
<tr>
<td>1:1</td>
<td>1.79 (1.29, 2.48)</td>
<td>0.86 (0.69, 1.07)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.67 (0.47, 0.96)</td>
<td>0.27 (0.14, 0.52)</td>
</tr>
<tr>
<td>Morphine/KRM-II-81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>1.88 (1.50, 2.36)</td>
<td>1.23 (0.83, 1.83)</td>
</tr>
<tr>
<td>1:1</td>
<td>1.44 (1.25, 1.65)</td>
<td>0.87 (0.51, 1.50)</td>
</tr>
<tr>
<td>3:1</td>
<td>0.83 (0.72, 0.95)</td>
<td>0.56 (0.42, 0.74)</td>
</tr>
<tr>
<td>Morphine/NS16085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>5.37 (4.26, 6.76)</td>
<td>3.98 (3.19, 4.96)</td>
</tr>
<tr>
<td>1:1</td>
<td>4.07 (3.06, 5.40)</td>
<td>2.46 (1.95, 3.10)</td>
</tr>
<tr>
<td>3:1</td>
<td>2.60 (2.05, 3.30)</td>
<td>1.88 (1.33, 2.65)</td>
</tr>
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</table>
Figure 16: Effects of drug combinations of oxycodone and GABA\_A PAMs (midazolam (left), NS15085 (center), and KRM-II-81 (left) on the analgesic effects (squares) in the von Frey assay. The rate of food-maintained operant responding (circles) in rats trained under a fixed ratio 10 schedule of food presentation was also plotted for comparison. Ordinate, percentage effect in correspondence to percentage of control responding rate (circles) or percentage of
maximal possible effect (squares); Abscissa, dose of drug (mg/kg). Filled gray squares indicate p < 0.05.
Figure 17: Effects of oxycodone in combination with GABA$_A$ PAMs (midazolam (left), NS15085 (center), and KRM-II-81 (left) on CFA-induced mechanical hyperalgesia (top panel) and thermal hyperalgesia (bottom panel). Ordinate, ED50 value (95% CL) of midazolam (left), NS16085 (center), and KRM-II-81 (right) expressed in mg/kg; ED50 value (95% CL) of oxycodone (mg/kg).
Table 4: Expected additive ED₅₀ values (Z_add), actual (experimentally determined ED₅₀ values (Z_mix), and the ratio of expected/actual ED₅₀ values for drug combinations for mechanical antinociception in CFA-treated rats

<table>
<thead>
<tr>
<th>Combination</th>
<th>Relative dose (ratio)</th>
<th>Z_add (95% CL)</th>
<th>Z_mix (95% CL)</th>
<th>Ratio Z_add/Z_mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxycodone/Midazolam</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>2.61 (2.29, 2.94)</td>
<td>2.20 (1.50, 3.23)</td>
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<td>1.19</td>
</tr>
<tr>
<td>1:1</td>
<td>2.06 (1.81, 2.315)</td>
<td>1.43 (1.13, 1.79)*</td>
<td></td>
<td>1.44</td>
</tr>
<tr>
<td>3:1</td>
<td>1.61 (1.23, 2.11)</td>
<td>1.56 (1.38, 1.76)</td>
<td></td>
<td>1.03</td>
</tr>
<tr>
<td>Oxycodone/KRM-II-81</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>2.97 (2.57, 3.37)</td>
<td>3.61 (3.04, 4.29)</td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td>1:1</td>
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<td>1.75 (1.32, 2.32)</td>
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<tr>
<td>3:1</td>
<td>1.75 (1.28, 2.39)</td>
<td>1.69 (1.50, 1.89)</td>
<td></td>
<td>1.04</td>
</tr>
<tr>
<td>Oxycodone/NS16085</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>7.27 (6.48, 8.06)</td>
<td>6.60 (4.17, 10.5)</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>1:1</td>
<td>5.61 (5.05, 6.17)</td>
<td>5.14 (3.24, 8.15)</td>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>3:1</td>
<td>3.13 (2.80, 3.46)</td>
<td>3.13 (2.45, 4.00)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Morphine/Midazolam</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>3.17 (2.80, 3.54)</td>
<td>2.48 (1.82, 3.38)</td>
<td></td>
<td>1.28</td>
</tr>
<tr>
<td>1:1</td>
<td>3.15 (2.79, 3.51)</td>
<td>3.65 (2.63, 5.05)</td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td>3:1</td>
<td>3.17 (2.81, 3.52)</td>
<td>2.77 (1.94, 3.95)</td>
<td></td>
<td>1.14</td>
</tr>
<tr>
<td>Morphine/KRM-II-81</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>3.53 (3.14, 3.92)</td>
<td>2.44 (1.95, 3.05)*</td>
<td></td>
<td>1.45</td>
</tr>
<tr>
<td>1:1</td>
<td>3.40 (3.03, 3.77)</td>
<td>2.62 (2.28, 3.02)*</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>3:1</td>
<td>3.31 (2.95, 3.68)</td>
<td>3.03 (2.64, 3.48)</td>
<td></td>
<td>1.09</td>
</tr>
<tr>
<td>Morphine/NS16085</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:3</td>
<td>7.87 (7.07, 8.68)</td>
<td>5.91 (4.64, 7.52)</td>
<td></td>
<td>1.33</td>
</tr>
<tr>
<td>1:1</td>
<td>6.29 (5.65, 6.93)</td>
<td>5.41 (4.07, 7.19)</td>
<td></td>
<td>1.16</td>
</tr>
<tr>
<td>3:1</td>
<td>5.00 (3.94, 6.35)</td>
<td>4.76 (4.28, 5.24)</td>
<td></td>
<td>1.05</td>
</tr>
</tbody>
</table>

* Indicates Z_mix confidence limits do not overlap with Z_add confidence limits and Z_mix is lower than Z_add (supra-additivity).
Figure 18: Effects of drug combinations of morphine and GABA<sub>A</sub> PAMs (midazolam (left), NS15085 (center), and KRM-II-81 (left) on the analgesic effects (squares) in the von Frey assay. The rate of food-maintained operant responding (circles) in rats trained under a fixed ratio 10 schedule of food presentation was also plotted for comparison. Ordinate, percentage effect in correspondence to percentage of control responding rate (circles) or percentage of maximal...
possible effect (squares); Abscissa, dose of drug (mg/kg). Filled gray squares indicate $p < 0.05$ (for analgesia) and * indicate $p < 0.05$ (for food-maintained operant responding).
Figure 19: Effects of morphine in combination with GABA<sub>A</sub> PAMs (midazolam (left), NS15085 (center), and KRM-II-81 (left) on CFA-induced mechanical hyperalgesia (top panel) and thermal hyperalgesia (bottom panel). Ordinate, ED50 value (95% CL) of midazolam (left), NS16085 (center), and KRM-II-81 (right) expressed in mg/kg; ED50 value (95% CL) of morphine (mg/kg).
CHAPTER 3 - COMBINATION THERAPY

DISCUSSION

The main finding of this study was that GABA\(\text{A}\) receptor PAMs; midazolam, KRM-II-81, and NS16085 produced significant mechanical analgesia in a model of chronic inflammatory pain. Additionally, these GABA\(\text{A}\) receptor PAMs are suitable for combination therapy with opioids such as oxycodone and morphine. Combinations of GABA\(\text{A}\) receptor PAMs and the commonly prescribed opioid, oxycodone produced mainly additive interactions. Similarly, mainly additive interactions were observed in combinations of GABA\(\text{A}\) receptor PAMs and another commonly prescribed opioid, morphine. It is important to note that while there were a few supra-additive (synergistic) effects, there were no infra-additive (antagonistic) effects observed in any of the fixed ratios between the GABA\(\text{A}\) PAMs and opioids. However, differences between the nonselective GABA\(\text{A}\) PAM midazolam and \(\alpha_2/\alpha_3\) subtype-selective GABA\(\text{A}\) receptor PAMs were observed in food-maintained operant responding. While rate-suppressing effects were observed when midazolam was combined with either oxycodone or morphine, rate-suppressing effects were not observed when opioids were combined with \(\alpha_2/\alpha_3\) subtype-selective GABA\(\text{A}\) receptor PAMs. Thus, \(\alpha_2/\alpha_3\) subtype-selective GABA\(\text{A}\) receptor PAMs may be more suitable for combination therapy than nonselective benzodiazepines, such as midazolam.

CFA-induced hyperalgesia is a well-characterized and well-recognized animal model of chronic inflammatory pain. Previous studies have shown CFA injection into the hindpaw of a rat to induce both mechanical and thermal hyperalgesia that is maintained for weeks (Nagakura et al., 2003). When drugs were administered alone morphine, midazolam, oxycodone, KRM-II-81 and NS16085 all dose-dependently attenuated CFA-induced mechanical hyperalgesia in the Von
Frey assay, which is in accordance with previous studies from our lab (as outlined in Chapter 2B of this dissertation, and (Thorn et al., 2015). In the Hargreaves test, morphine, midazolam, and oxycodone dose-dependently attenuate thermal hyperalgesia, while KRM-II-81 and NS16085 failed to attenuate thermal hyperalgesia. This finding is in accordance with previous studies in our lab that were discussed in Chapter 2 of this dissertation.

While benzodiazepines and opioids are prescribed simultaneously in some clinical settings, there are guidelines advising against this practice. The reason being that both medications increases overdose risk (Kim, McCarthy, Hoppe, Mark Courtney, & Lambert, 2018). A recent study, found the combination of the opioid fentanyl and the benzodiazepine midazolam was diagnostic for in-hospital deaths following serious medical illness and interventions that included the two drugs (Saad et al., 2018). Despite this caveat, a recent study found that the co-prescribing rate of benzodiazepines and opioids has increased over time (Rhee, 2018). Therefore, the suitability of combination therapy regarding benzodiazepines and opioids should be studied more extensively. Specifically, combination therapy studies assessing potential analgesic effects.

Until now, there was limited preclinical evidence that benzodiazepines are analgesics and there is still little clinical evidence. Previous studies have demonstrated that this is probably due to the fact that the analgesic actions of benzodiazepines are most likely masked by other effects such as sedation, cognitive impairment, and muscle relaxation (Zeilhofer, Möhler, & Di Lio, 2009). Due to the lack of literature assessing benzodiazepine-opioid interactions, as well as the increase in co-prescribing rate of benzodiazepines and opioids, the present study sought to study the analgesic effects of oxycodone in combination with GABA\textsubscript{A} PAMs: midazolam, KRM-II-81, and NS16085. To do so, a dose-additional analysis was used. This is a
powerful and systematic method of examining pharmacological interactions that has frequently been used in our laboratory to conduct drug-drug interaction studies (Tallarida, 2001). Our findings demonstrate that α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs KRM-II-81 and NS16085 produced mainly additive and few supra-additive interactions with both oxycodone and morphine for attenuating mechanical hyperalgesia. These effects are apparent across a wide range of drug proportions, from 25% GABA<sub>A</sub> PAM (3:1) to 75% GABA<sub>A</sub> PAM (1:3).

Additionally, the same doses that produced analgesic effects, did not significantly reduce the rate of operant responding for food reward, which is considered a measure of behavioral suppression. The nonselective midazolam also produced additive to supra-additive interactions with both oxycodone and morphine for decreasing mechanical hyperalgesia (across all drug proportions). However, under some drug proportions, there were rate-altering effects (suppression) observed in the food-maintained operant responding. This finding suggests that α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs may be more suitable for combination therapy than classical, nonselective benzodiazepines such as midazolam.

Collectively, these findings further support the notion that α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs have the potential to be clinically effective, in that not only can these compounds manage pain alone, but when combined with opioids they are able to produce at least additive effects, and even supra-additive effects under some drug combinations. Opioid abuse is now the primary cause of accidental deaths in the United States (Saad et al., 2018). Combining the two classes of drugs may minimize adverse effects, such as abuse-related effects, due to the lower doses that are used of each of drug, while simultaneously maintaining the therapeutic effect of both compounds.
CHAPTER 4 – BEHAVIORAL EFFECTS OF SUBTYPE-SELECTIVE GABA$_A$ PAMS

INTRODUCTION

Spinal GABA$_A$ receptors have been shown to play a major role in the perception of pain (Zeilhofer et al., 2015). Previous studies have indicated that diminished glycinergic and GABAergic mediated-inhibition in the spinal cord, is a major contributing factor to inflammatory and neuropathic chronic pain (Di Lio et al., 2011; Munro et al., 2008). Thus, pharmacological restoration of GABAergic synaptic inhibition is a promising solution for pain management. As a result, GABA$_A$ receptors have received increasing attention as pharmacological targets for pain control.

There is considerable evidence implicating that different $\alpha$ subtypes of the GABA$_A$ receptor are associated with different actions of the benzodiazepine site of the GABA$_A$ receptor (Zeilhofer, 2009; Ralvenius et al., 2015; Zeilhofer et al., 2015). For example, the $\alpha_1$ subtype has been shown to mediate sedative and abuse-related effects, $\alpha_2/\alpha_3$ subtypes are thought to mediate analgesic and anxiolytic effects, and the $\alpha_5$ subtype has been associated with cognition. As a result, subtype-selective GABA$_A$ receptor positive allosteric modulators are being developed as candidate analgesics. However, compounds that are truly selective for $\alpha_2/\alpha_3$ subtype-containing GABA$_A$ receptors are scarce. The majority of reported subtype-selective GABA$_A$ receptors also display preference for $\alpha_5$ subtype-containing GABA$_A$ receptors. This poses a problem because the $\alpha_5$ subtype is thought to play a key role in cognitive control. Since $\alpha_5$ GABA$_A$ receptor negative allosteric modulators (NAMs) enhance cognition, PAMs selective for $\alpha_5$-subtypes could possibly impair cognition (Atack, 2010). Previous studies have reported KRM-II-81 and NS16085 as $\alpha_2/\alpha_3$ subtype-selective GABA$_A$ receptor PAMs (de Lucas et al., 2015; Lewter et
al., 2017). However, the relationship between the analgesic effects and other off-target pharmacological effects of these compounds remains underexplored. Therefore, this study sought to study various behavioral effects and the selectivity profile of α2/α3 subtype-selective GABAÅ PAMs: KRM-II-81 and NS16085.
CHAPTER 4A – BEHAVIORAL EFFECTS OF SUBTYPE-SELECTIVE GABA\textsubscript{A} PAMS

MATERIALS AND METHODS

Subjects: Described in Chapter 2B.

Induction of inflammatory pain: Described in Chapter 2B.

Mechanical hyperalgesia: Described in Chapter 2B.

Schedule-controlled responding: Described in detail in Chapter 3.

During test sessions, rats received a single injection of KRM-II-81, NS16085, and midazolam and then were immediately placed in the operant chamber and the program was started.

Place escape/avoidance paradigm: The affective-emotional component of pain was studied using the place escape/avoidance paradigm (Fuchs & McNabb, 2012; LaBuda & Fuchs, 2000). In this paradigm, rats were placed into an inverted rat cage (26 × 47 × 22 cm) that was painted half white and half black, as described previously, (Li et al., 2014). The cage was placed on an elevated grid so the experimenter could apply the plantar surface of the hind paw with a 60 g von Frey filament (this was done every 15s over the course of a 30 min test). When the rat was on the white side of the cage, the von Frey filament was applied to the non-inflamed paw. When the rat was on the black side of the cage, the von Frey filament was applied to the inflamed right paw. The location of the rat in the chamber (black vs. white) at the time of the mechanical stimulation was observed. Percentage of time the rats spent on the white side of the chamber was calculated from observations that were made every 15 s during each 5 min period, and was used as the indication of escape/avoidance learning (number of observations of rats on the white side/total number of observations × 100). To examine the effects of GABA\textsubscript{A} PAMs on the place escape/avoidance learning behaviors, KRM-II-81, or vehicle was administered 10 min before the
test session. This was a blind experiment, in which the experimenter was unaware of the treatment and dose of the subjects.

**Horizontal Wire Test:** The horizontal wire test was used to measure myorelaxant activity, as previously described (Bonetti et al., 1982). Rats (n=10/group) were slightly restrained and lifted by the tail and allowed to grasp a wire (galvanized, 16 gauge 1/16” diameter) that was horizontally strung across a rat cage (26 × 47 × 22 cm) with their forepaws. The inability to complete this task (within 3 s) was recorded every 20 min after injection of drug. A cumulative-dosing procedure was used in this test, where measurements were recorded every 20 minutes, and immediately after each measurement, rats received the next dose of drug. This was a blind experiment, in which the experimenter was unaware of the treatment and dose of the subjects.

**T-maze Spontaneous Alternation Task:** T-maze spontaneous alternation experiments were conducted in a custom-made, grey painted Plexiglas T-maze. The start arm was 50 cm long and 16 cm wide, the goal arms 50 cm long and 10 cm wide, and the wall height of the maze was 30 cm tall. Guillotine doors (positioned at the midpoint of each arm) were cut to fit the maze to prevent the rat from retreating after a choice was made. Different groups of rats (n=10/group), were pre-treated with KRM-11-81, NS16085, midazolam, or saline, 20 minutes prior to the first trial (total of 7 trials per test). A trial began once a rat was placed in the start arm (facing away from the goal arms). Upon leaving the start arm, rats were allowed to choose either goal arm (right or left). Once a goal arm was chosen (defined by the presence of the rat’s body and tail within it), the guillotine door was quietly slid down in order to confine the rat for 30 s. After 30 s, the rat was removed and placed back in its home cage and left for 30 s before being placed back in the start arm, starting the next trial. This task consisted of seven consecutive trials, if a
rat could not complete a trial within two minutes, only the previous trials were used to calculate the alternation score. This was a blind experiment, in which the experimenter was unaware of the treatment and dose of the subjects.

**Data Analysis:** All graphs and statistical analyses were performed with the GraphPad Prism 7.0 program (GraphPad Software, San Diego, CA, USA). The mean values (± SEM) were calculated from individual animals for mechanical and thermal nociception assays, as well as PEAP. One-way or two-way repeated measurements ANOVA, followed by post hoc Bonferroni’s tests, were used to determine the statistical significances. Critical values for the main or interaction effects were set at p< 0.05.

In Figure 21, mechanical analgesic effects were normalized and quantified for each rat as % maximal possible effect (MPE) for each drug dose. The following equation was used to quantify % MPE: \( %\text{MPE} = \left( \frac{\text{Post-drug value for a behavioral response} - \text{Pre-drug value for a behavioral response}}{\text{Pre manipulation [CFA or CCI] value} - \text{Pre-drug value for a behavioral response}} \right) \times 100 \). Myorelaxant activity is presented as the percentage of rats unable to grasp the wire and \( \chi^2 \) Analysis and Fisher’s exact tests were used to determine statistical significance. The alternation score in the T-maze is presented as the percentage of alternation. The alternation score % of each rat was measured using the following equation: \( (\# \text{ of alternations}/n-1) \times 100 \), where “n” is the number of trials. One-way ANOVA was used to determine statistical significance. Rate of operant responding is presented as a percentage of the control response rate, as previously described (Thorn et al., 2015). The percentage of control response rate data (Figure 21) was analyzed using one-way ANOVA followed by post hoc Bonferroni’s test. Since the analgesic effects of the drugs were observed 45-60 min after drug administration, only data from
the third cycle of each test session was presented for comparison.

**Drugs:** *Described in Chapter 2.*

All drugs were given via intraperitoneal injections. All doses are expressed as mg weight free base per kg body weight.
CHAPTER 4A – BEHAVIORAL EFFECTS OF SUBTYPE-SELECTIVE GABA\textsubscript{A} PAMS

RESULTS

The effect of one of the subtype-selective GABA\textsubscript{A} PAMs, KRM-II-81, on affective pain was assessed using the placed escape-avoidance paradigm (PEAP), 24 h after CFA treatment (Figure 20). In rats that received saline treatment, when repetitive mechanical stimuli were applied, rats switched from not having a preference to either side, to a preference to the white side (where the non-injured hind paw was stimulated), demonstrating the place escape/avoidance behavior. Two-way ANOVA revealed a significant dose × time interaction ($F_{[10, 70]} = 6.50, p < 0.05$). Post hoc analyses found that 1.0 mg/kg and 3.2 mg/kg KRM-II-81 significantly decreased the place escape/avoidance behavior from 15-30 min of the session (25-40 min after the drug was administered), when compared to saline-treated animals.

The response rate of food-maintained responding and myorelaxant effects of $\alpha$2/$\alpha$3 subtype-selective GABA\textsubscript{A} PAMs were tested in pain-free animals (Figure 21). When vehicle was administered, the response rate for each rat (expressed as a percentage of its response rate on the three preceding control days), was $100.6 \pm 4.08\%$ for midazolam vehicle, $108.9 \pm 5.71\%$ for the KRM-II-81 vehicle, and $100.4 \pm 4.42\%$ for the NS16085 vehicle. There was no significant change over the six response periods, within a test period (repeated one-way ANOVA, midazolam group: $F_{[5, 30]} = 1.29, p > 0.05$, KRM-II-81 group: $F_{[5, 30]} = 1.16, p > 0.05$, NS16085 group: $F_{[4, 20]} = .331, p > 0.05$). Midazolam dose-dependently reduced responding rate when repeated one-way ANOVA was performed ($F_{[4, 20]} = 10.71, p < 0.05$). Post hoc analyses revealed significant rate-suppressing effects of midazolam at doses 5.6 and 10 mg/kg, compared to vehicle. Neither KRM-II-81 nor NS16085 significantly reduced the response rate.
up to 10 mg/kg KRM-II-81 and 32 mg/kg NS16085, which are doses much higher than the doses
that produce antinociception. In the horizontal wire task, when given vehicle, the myorelaxant
activity (expressed as a percentage of rats unable to grasp the wire) was 0% for all GABA_A
PAMs: midazolam, KRM-II-81, and NS16085. Midazolam and KRM-II-81 produced a dose-
dependent impairment of the grasping reflex ($\chi^2 = 18.3$, $p < 0.05$ for midazolam; $\chi^2 = 27.02$, $p <$
0.05 for KRM-II-81). Post hoc analyses revealed doses of 10 mg/kg midazolam, 17.8, and 32
mg/kg KRM-II-81 significantly increased myorelaxant activity when compared to their
respective vehicles. NS16085 failed to produce myorelaxant activity up to 56 mg/kg NS16085.
For comparison purposes, data from the mechanical nociception assay (in Chapter 2C) were
converted into MPE% and were also shown in Figure 21.

The effects of $\alpha_2/\alpha_3$ subtype-selective GABA_A PAMs on cognition were tested in the
spontaneous alternation T-maze task (Figure 22). When saline was administered, the mean
alternation percentage was 70 ± 7.5 %. When compared with saline, post hoc analyses revealed a
dose of 5.6 mg/kg midazolam, significantly reduced the spontaneous alternation percentage to
33.3 ± 5.14 %. Within the dose range that antinociception was produced, neither KRM-II-81
(alternation percentage = 61.1%) nor NS16085 (alternation percentage = 63.9%) pretreatment
had a significant effect on the spontaneous alternation score in the T-maze task.
**Figure 20:** The effect of KRM-II-81 on the mean percentage of time (±SEM) spent in the white side of the chamber during the session (*n*=8/group). Rats were stimulated on the right (CFA-treated) paw with a 60 g von Frey filament when in the black side of the chamber, and stimulated on the left paw (untreated) when in the white side of the chamber. Filled-in symbols (gray) indicate time points that are significantly different from vehicle treatment condition (*p* <0.05). Ordinates, percentage of time on the white side as measured by PEAP; Abscissa, time (minutes) in 5 min intervals over the 30 minutes session.
Figure 21: Percentage of maximum possible effects of GABA<sub>A</sub> PAMs on CFA-induced mechanical nociception (square symbol, n=6/group), muscle relaxant activity, (triangle symbol, n=10/group), and percentage of control responding rate (circle symbol, n=6-8/group). Filled-in symbols (gray) indicate time points that are significantly different from vehicle treatment condition (p <0.05).

Ordinates: percentage of maximal possible effects or percentage of control responding rate; Abscissa, doses (mg/kg, i.p.) of midazolam, KRM-II-81 (middle), and NS16085 (bottom).
Figure 22: Acute effects of GABA$_A$ PAMs on cognition (working memory) in rats. Ordinates: percentage of alternation; Abscissa, doses (mg/kg, i.p., $n=10$/group) of KRM-II-81 (5.6 KRM-II-81), and NS16085 (17.8 mg/kg), midazolam (5.6 mg/kg). Asterisks indicate alternation percentage scores significantly different from the saline-treated group ($p<0.05$).
The primary finding of the current study was that α2/α3 subtype-selective GABA_A PAMs KRM-II-81 and NS16085 displayed a favorable side-effect profile, when compared to the nonselective benzodiazepine midazolam. Both KRM-II-81 and NS16085 were able to selectively produce analgesic effects. Whereas, midazolam displayed cognitive dysfunction, analgesia, rate-suppression, and muscle-relaxant effects at similar doses. In addition, KRM-II-81 dose-dependently attenuated the place escape/avoidance behavior, which measures the affective component of pain. Collectively, these data support the notion that α2/α3 subtype-selective GABA_A PAMs can produce analgesia without producing other pharmacological effects that are associated with benzodiazepines; further confirming their subtype-selectivity.

Chronic pain is a multidimensional phenomenon. The majority of pain research focuses on the sensory-discriminative component of pain, while the affective-motivational component of chronic pain is underexplored. The place/escape avoidance paradigm, is effective for measuring the affective-motivational component of pain in rodents (LaBuda & Fuchs, 2000). Therefore, in the present study, we examined the effect of KRM-II-81 on the affective-motivational component of chronic pain, using PEAP (Figure 4). KRM-II-81 significantly decreased the place/escape avoidance behavior. This finding is consistent with a study done by Knabl et al., (2008) which reported α1-sparing benzodiazepine-site ligand L-838,417 as able to reduce the
activity of brain areas related to the associative-emotional component of pain (Knabl et al., 2008).

In addition, to pain-related behavioral assays, we sought to test other behavioral effects (in pain-free animals) that would confirm the subtype-selectivity of KRM-II-81 and NS16085 (Figure 5). Previous studies suggest that sedative properties of benzodiazepine-like drugs are primarily mediated through the α1 subtype (McKernan et al., 2000; Rudolph et al., 1999). A significant decrease in response-rate could be indicative of general behavioral disruption and/or sedation.

Therefore, we first examined the effects of the α2/α3 GABA_A PAMs on operant food-maintained behavior. We found that 5.6 and 10 mg/kg of midazolam significantly decreased the percentage of control responding of pain-free animals. Within the dose-range that produced significant analgesia, neither KRM-II-81 nor NS16085 had a significant effect on response-rate. Stable response-rates in KRM-II-81 and NS16085-treated rats confirms their lack of selectivity for α1 subtype-containing GABA_A receptors. Next, we sought to study the myorelaxant effects of KRM-II-81, NS16085, and midazolam. The α2/α3 subtypes of the GABA_A receptor have been associated with myorelaxant activity (Crestani et al., 2001). We found doses of 10 mg/kg midazolam and 17.8 and 32 mg/kg KRM-II-81 significantly increased the percentage of rats unable to grasp the wire, (indicating myorelaxant activity) when compared to their respective vehicles. NS16085 failed to produce myorelaxant activity up to 56 mg/kg NS16085. Therefore, only midazolam produced myorelaxant effects within the dose range that analgesic effects were observed.

Lastly, we studied the effects of GABA_A PAMs on cognition. Genetic and
pharmacological studies have suggested that α5-containing GABA_A receptors mediate the cognitive dysfunction that is associated with benzodiazepine-use (Atack, 2010; Savic et al., 2009). The T-maze has been well characterized and is shown to measure deficits of working/spatial memory in rodents (Deacon & Rawlins, 2006; Forster, Prather, Patel, & Lal, 1995). A study done by Borde et al., (1997) showed diazepam to induce cognitive dysfunction via the alternation T-maze (Borde, Krazem, Jaffard, & Béracochéa, 1997). Using a similar approach, this study sought to compare the percentage of alternation of rats that received GABA_A receptor PAMs (at doses that produced maximal antinociception), and saline treated rats. We found that only midazolam pretreatment significantly decreased the mean percentage of alternation, when compared to rats that received saline. KRM-II-81, and NS16085 did not significantly affect the alternation percentage observed in the T-maze. The α1-subtype also may contribute to cognitive dysfunction of benzodiazepines (Savic et al., 2009). Therefore, it is difficult to conclude how much of the midazolam-induced cognitive dysfunction was mediated by its selectivity at the α1-subtype.

In conclusion, the behavioral effects of KRM-II-81 and NS16085 reported in this study confirm the lack of efficacy at the α1 and α5 subtypes of GABA_A receptors. Both KRM-II-81 and NS16085 demonstrated behavioral specificity and a favorable side-effect profile when compared to nonselective GABA_A PAM midazolam (as indicated by their ability to selectively produce analgesic effects). Collectively, these data support the notion that α2/α3-subtype selective GABA_A PAMs are a viable novel class of drugs for the management of chronic pain.
CHAPTER 4B – DISCRIMINATIVE STIMULUS EFFECTS

INTRODUCTION

Benzodiazepines are currently used to manage an array of conditions such as anxiety, panic attacks, insomnia, seizures, and alcohol withdrawal (Griffin, Kaye, Bueno, & Kaye, 2013; Nordqvist, 2018). While acute use of benzodiazepines are viewed as effective and relatively safe, long-term use may lead to adverse effects such as tolerance and dependence.

Emerging evidence suggests that different α subunits of GABA_A receptors are associated with different pharmacological effects associated with benzodiazepine-use. For example, the α1-subtype is thought to mediate sedative and abuse-related effects, α2/3 subtypes mediate anxiolytic and analgesic effects, and the α5 subtype is associated with cognition (Collinson et al., 2002; Engin, Liu, & Rudolph, 2012; Löw et al., 2000; R. M. McKernan et al., 2000; Mohler, 2009; Platt et al., 2005). This finding has led to the development of various α-selective and α-sparing compounds to enhance the efficacy and minimize the side-effect profile of candidate therapeutics.

Benzodiazepines, act as positive allosteric modulators (PAMs), in that they increase GABA neurotransmission by interacting with the benzodiazepine binding site of GABA_A receptors. Spinal GABA_A receptors have been shown to play a key role in pain processing. A decrease in GABAergic inhibitory tone is observed in rodent models of inflammatory and neuropathic pain (Zeilhofer et al., 2009). Thus, restoration of GABAergic-induced inhibition is a plausible solution to managing chronic pain. Benzodiazepines are not suitable for treating chronic pain because their activity at α1, α2, α3, and α5 subtypes of the GABA_A receptor often produces pharmacological effects unrelated to analgesia. Whereas, positive allosteric
modulators that are selective for α2/α3 subtype-containing GABA_\textsubscript{A} receptors can produce antinociceptive effects (a α2/α3 subtype-mediated effect) without producing other pharmacological effects that are associated with nonselective benzodiazepines.

Previous studies have reported KRM-II-81 and NS16085 as α2/α3 subtype-selective GABA_\textsubscript{A} receptor PAMs \textit{in vitro} (de Lucas et al., 2015; Lewter et al., 2017). Preliminary \textit{in vivo} studies have shown both compounds to be effective in attenuating pain, in models of visceral pain, inflammatory, neuropathic, and capsaicin-induced pain (Chapter 2). However, the \textit{in vivo} pharmacological activity at the α1-subtype, has yet to be explored using these α2/α3 subtype-selective GABA_\textsubscript{A} receptor PAMs. Additionally, little is known about the drug-abuse related effects of KRM-II-81 and NS16085. Therefore, in order to confirm the lack of in vivo activity at the α1- subunit and to explore the drug abuse related effects of these compounds, we studied the discriminative stimulus effects of KRM-II-81 and NS16085 using the drug discrimination procedure.
CHAPTER 4B – DISCRIMINATIVE STIMULUS EFFECTS
MATERIALS AND METHODS

Subjects: Described in Chapter 2B.

Drug Discrimination: Drug Discrimination studies were conducted using commercially available two-lever operant chambers placed within sound-attenuating, ventilated enclosures (Coulbourn Instruments Inc., Allentown, PA, USA) as described previously (Siemian et al., 2017; Qiu et al., 2014, 2015). Data were collected through an interface using the Graphic State 3.03 software (Coulbourn Instruments Inc., Whitehall, PA, USA). Previously described training protocols were used (Li et al., 2008; Qiu et al., 2015). Rats were trained to discriminate 3.2 mg/kg midazolam injected intraperitoneally (i.p.) from saline in a multiple-cycle procedure. Another group of rats were trained to discriminate 3.2 mg/kg KRM-II-81 from its vehicle. Each cycle consisted of a 15-min timeout during which the chamber was dark and responses had no programmed consequence, followed by a 5-min response period, during which a house light and a cue light above each lever were illuminated and signaled availability of reinforcement. Ten consecutive responses (fixed ration [FR] 10) on the correct lever resulted in food deliver (45 mg; BioServ Inc., Frenchtown, NJ, USA). The correct lever was predetermined by an injection (e.g., right, saline; left, midazolam). Response periods ended after 5 min or after deliver of 10 food pellets, whichever occurred first.

Training began with single-cycle sessions, in which 3.2 mg/kg midazolam, 3.2 mg/kg KRM-II-81, or their respective vehicles were administrated immediately before the start of the session. Sessions were conducted daily (7 days per week) according to a double alternation schedule (i.e., saline, saline, drug, drug). Rats had to achieve at least 90% of the total responses
on the correct lever for either five consecutive sessions or six out of seven sessions in order to progress to multiple-cycle training. For multiple-cycle training, some training days consisted of two cycles in which either the vehicle lever or the training drug (midazolam or KRM-II-81) lever was active during both cycles, depending on the injection given 10 min prior to the initiation of the training sessions. Other training days consisted of one to three vehicle training cycles preceding the administration of training drug and two training drug cycles. Other training cycles consisted of five vehicle training days. This training protocol minimizes the subjects’ lever bias. These protocols were varied; rats needed to pass two consecutive sessions (one vehicle training session and one training drug session) by responding at least 90% on the correct lever during each active period prior to each test.

Test sessions lasted up to five cycles and were identical to training sessions except that 10 consecutive responses on either lever delivered a food pellet. During test sessions, vehicle or test drugs were administered before the start of the first cycle. Control dose-effect curves of the training drug were periodically established throughout the study in order to assess the performance stability of midazolam discrimination. In substitution studies, compounds with (flunitrazepam) or without (morphine, methamphetamine) known GABA_A receptor activity were tested in order to examine the generality of the discriminative stimulus effects of midazolam.

**Drugs:** Described in Chapter 2.

All drugs were given via intraperitoneal injections. All doses are expressed as mg weight free base per kg body weight.

**Data Analysis:** Data were expressed as the percent of midazolam-associated lever responding, and as the rate of responding within the test session. Since the analgesic effects of the GABA_A
PAMs; KRM-II-81 and NS16085 reached a maximum of 45-60 min after drug administration, dose-effect curves were constructed using data from the third cycle of each test session (55-60 min after drug administration). A cumulative dosing procedure was used for shorter-acting drugs such as midazolam, flunitrazepam, morphine, and methamphetamine.

Data were analyzed by repeated-measures analyses of variance (ANOVA) followed by post hoc Bonferonni’s tests. Critical values for the main or interaction effects were set at $p < 0.05$. In generalization/substitution studies, full substitution was defined as $> 80\%$ drug-appropriate responding; partial substitution was defined as $40-80\%$ drug-appropriate responding; no substitution was defined as $< 20\%$ drug-appropriate responding. Data are presented as the mean and standard error of the mean (SEM).
CHAPTER 4B – DISCRIMINATIVE STIMULUS EFFECTS

RESULTS

In the rats trained to discriminate midazolam (3.2 mg/kg) from its vehicle, all rats produced reliable discrimination in 30 sessions (Figure 23). In the last session of training, the mean percentage responding on the midazolam-associated lever was 100% and the mean response rate was 0.70 ± 0.09 responses/sec. When rats were re-trained at a lower dose of midazolam (0.56 mg/kg), reliable discrimination was observed after 22 sessions. In the last session of re-training, the percentage responding on the midazolam-associated lever was 100% and the mean response rate was 0.83 ± 0.06 responses/sec. In another group of rats, trained to discriminate α2/α3 subtype-selective GABA\(_A\) PAM KRM-II-81 from its vehicle, reliable discrimination was not obtained. After 115 training sessions, the rats training to discriminate KRM-II-81 (3.2 mg/kg) did not meet the performance criteria, and that group was terminated.

Midazolam dose-dependently increased midazolam-lever responding in rats trained to discriminate the high dose (3.2 mg/kg) and low dose (0.56 mg/kg) of midazolam (Figure 24). Under the higher training dose condition, a cumulative dose of 3.2 mg/kg midazolam produced > 80% of total responses occurring on the midazolam-lever. Under the low training dose condition, a cumulative dose of 1.0 mg/kg midazolam produced > 80% of total responses occurring on the midazolam-lever. These doses of midazolam did not significantly decrease the response rate, when compared to vehicle pre-treatment. The benzodiazepine site antagonist flumazenil, antagonized midazolam, shifting midazolam dose-response curves to the right in rats under the low (2.31-fold rightward shift) and high (2.43-fold rightward shift) training conditions (Figure 24).
The classical benzodiazepine flunitrazepam fully substituted midazolam-lever responding. Under the low training dose condition, cumulative doses of 0.32 and 0.56 mg/kg flunitrazepam produced > 80% of total responses occurring on the midazolam lever (Figure 25). Under the high training dose, the dose of 1.78 mg/kg flunitrazepam produced > 80% of total responses occurring on the midazolam lever. Flunitrazepam did not significantly reduce the response rate. Neither morphine nor methamphetamine significantly increased midazolam-lever responding under both midazolam training conditions (< 20% of total responses occurring on the midazolam lever). *Post hoc* analyses revealed that 10 mg/kg of morphine, and 10 mg/kg of methamphetamine significantly decreased the total responses.

The α2/α3 subtype-selective GABA_A PAM KRM-II-81 only partially substituted midazolam-lever responding (Figure 26). Under both midazolam training conditions, 32 mg/kg KRM-II-81 produced 40-80% of total responses occurring on the midazolam lever. Under the high training dose condition, *post hoc* analyses revealed that 32 mg/kg significantly increased midazolam-lever responding and significantly decreased the total responses, when compared to vehicle conditions. The α2/α3 subtype-selective GABA_A PAM NS16085 also only partially substituted midazolam-lever responding (40-80% of total responses occurring on the midazolam lever. NS16085 failed to significantly increase midazolam-lever responding. *Post hoc* analyses revealed that 100 mg/kg of NS16085 significantly decreased the total responses.
Figure 23: Training sessions of rats trained to discriminate 3.2 mg/kg of midazolam (black circles) and 3.2 mg/kg of KRM-II-81 (white squares) using a two-lever food-reinforced procedure.
Figure 24: Effects of midazolam alone (black-filled symbols) and in the presence of 5.6 mg/kg flumazenil (gray filled symbols) in rats trained to discriminate between midazolam (0.56 mg/kg - left panels; 3.2 mg/kg - right panels) and vehicle using a two-lever-food-reinforced procedure. The mean (± SEM) percentage of responses on the midazolam-associated lever (top panels) and the mean (± SEM) rate of responding (bottom panels). Points above “V” indicate vehicle. Each point represents the average (± SEM) of 6-8 rats.
Figure 25: Effects of flunitrazepam, morphine, and methamphetamine in rats trained to discriminate between midazolam (0.56 mg/kg – open triangles; 3.2 mg/kg – filled triangles) and vehicle using a two-lever-food-reinforced procedure. The mean (± SEM) percentage of responses on the midazolam-associated lever (top panels) and the mean (± SEM) rate of responding (bottom panels). Points above “V” indicate vehicle. Asterisks indicate significant differences in midazolam-lever responding or rate of responding (when compared to vehicle pretreatment), *p < 0.05.
Figure 26: Effects of α2/α3 subtype GABA<sub>A</sub> PAMs KRM-II-81 (left panels) and NS16085 (right panels) in rats trained to discriminate between midazolam (0.56 mg/kg –open triangles; 3.2 mg/kg –filled triangles) and vehicle using a two-lever-food-reinforced procedure. The mean (± SEM) percentage of responses on the midazolam-associated lever (top panels) and the mean (± SEM) rate of responding (bottom panels). Points above “V” indicate vehicle. Asterisks indicate significant differences in midazolam-lever responding or rate of responding (when compared to vehicle pretreatment), *p < 0.05.
The primary finding of this study was that reliable discrimination was not observed in rats that were trained to discriminate α2/α3 subtype-selective GABA\textsubscript{A} PAM KRM-II-81 from its vehicle. In addition, α2/α3 subtype-selective GABA\textsubscript{A} receptor PAMs KRM-II-81 and NS16085 only partially substituted midazolam-lever responding in rats trained to discriminate a high dose (3.2 mg/kg) and low dose (0.56 mg/kg) of midazolam. The doses that produced partial substitution were much larger than the doses that produced significant analgesic effects. Furthermore, while flunitrazepam (a benzodiazepine) fully substituted midazolam-lever responding, morphine and methamphetamine failed to significantly increase midazolam-lever responding. Since, the discriminative stimulus effects of midazolam are thought to be primarily mediated by α1-subtype GABA\textsubscript{A} receptors, these findings suggest KRM-II-81 and NS16085 have limited in vivo pharmacological activity at α1-subtype containing GABA\textsubscript{A} receptors.

Drug discrimination is a well-established procedure that can be used to explore the abuse-related effects of psychoactive drugs (Solinas, Panlilio, Justinova, Yasar, & Goldberg, 2006). Drug discrimination procedures in animals have a high predictive validity regarding the effects of treatments on drug self-administration, thus it is likely that drug discrimination has a good predictive validity for drug self-administration (Solinas, Panlilio, Justinova, Yasar, & Goldberg, 2006). The present study sought to gain insight on the drug-abuse potential of α2/α3 subtype-selective GABA\textsubscript{A} receptor PAMs by studying the discriminative stimulus effects of these compounds using the drug discrimination procedure. Therefore, the first objective of the present study was to train a group of rats to discriminate a high dose of midazolam (3.2 mg/kg) from its
vehicle. Rats achieved reliable discrimination after 30 sessions, once all test compounds were tested under this high training dose, rats were then re-trained to discriminate a lower dose of midazolam (0.56 mg/kg). Consistent with the literature, we found that rats achieved reliable discrimination at a similar rate as previous studies (Sannerud & Ator, 1995). For example, rats in the present study acquired 0.56 mg/kg midazolam discrimination after 22 sessions. In a study conducted by Gerak et al., rats acquired 0.56 mg/kg midazolam discrimination in 20.1 ±1.8 training sessions (Gerak et al., 2011). The next objective of the present study was to train a group of rats to discriminate 3.2 mg/kg KRM-II-81 from its vehicle. Reliable discrimination was not observed and the group was terminated after 115 training sessions. This suggests that the discriminative stimulus effects of α2/α3 subtype-selective GABA_A receptor PAMs may be weaker than nonselective GABA_A receptor PAMs, such as midazolam. This finding is consistent with a study that sought to study the discriminative stimulus effects of TPA023B (Kohut & Ator, 2008). TPA023B has in vitro antagonist efficacy at α1 subtypes and partial-agonist efficacy at α2/α3/α5 subtypes. The TPA023B training group showed no evidence of acquiring TPA023B discrimination after 160 sessions. These findings, along with the findings from the present study, confirm the primary involvement of the α1 subtype in discriminative stimulus effects of benzodiazepines.

To ensure that rats had achieved reliable discrimination, a series of generalization and substitution tests were performed. In the drug discrimination procedure, stimulus generalization (substitute) studies are typically used to determine whether a discriminative stimulus will substitute for other drugs. The reason being, if two drugs (test drug and training drug, respectively) produce similar subjective effects, though not necessarily an identical mechanism
of action, stimulus generalization will most likely occur (Young, 2009). In the present study, full substitution was defined as > 80% drug-appropriate responding; partial substitution was defined as 40-80% drug-appropriate responding and no substitution was defined as < 20% drug-appropriate responding. The classical benzodiazepine flunitrazepam fully substituted midazolam-lever responding, which is consistent with previous reports (Gerak et al., 2011). In contrast, morphine and methamphetamine, drugs that don’t act on the GABA_A receptor, failed to substitute midazolam-lever responding up to doses that markedly decreased response rates. This finding is consistent with the literature (Bai, France, & Gerak, 2011). Collectively, these data suggest that rats were well-trained and acquired reliable discrimination.

Lastly, we sought to study the discriminative stimulus effects of α2/α3 subtype-selective GABA_A PAMs KRM-II-81 and NS16085 by seeing whether KRM-II-81 or NS16085 could substitute for midazolam-lever responding. We found that KRM-II-81 and NS16085 only partially substituted midazolam-lever responding. This partial substitution of KRM-II-81 and NS16085 suggests that these compounds have limited in vivo pharmacological activity at α1-subtype containing GABA_A receptors. Furthermore, it is worth noting that this partial substitution is observed at doses that are much larger than the doses that produce significant analgesic effects. For example, while 3.2 mg/kg KRM-II-81 produces significant analgesic effects, partial substitution was not observed until rats were treated with 32 mg/kg KRM-II-81. A dose of 10 mg/kg NS16085 produces significant analgesic effects and partial substitution was observed when rats received 100 mg/kg. We also noticed markedly decreased response rates at 32 mg/kg KRM-II-81 and 100 mg/kg NS16085. It is possible that off-target effects are observed at these larger doses of α2/α3 subtype-selective GABA_A PAMs. It is likely that the partial
substitution and rate-suppressing effects are due to large doses of KRM-II-81 and NS16085 beginning to act on α1 subtype-containing GABA_A receptors.

Taken together, findings from this present study support the notion that α2/α3 subtype-selective GABA_A receptor PAMs have a more desirable side-effect profile than classical benzodiazepines in that these compounds have limited in vivo pharmacological activity at α1 subtype –containing GABA_A receptors, at doses that produce maximal analgesia. Since, the α1-subtype is associated with undesirable effects such as sedation and drug-abuse related effects, limited in vivo pharmacological activity at this subtype is ideal for candidate analgesics. In addition, reliable discrimination was not acquired in the group of rats that were trained to discriminate KRM-II-81 from its vehicle, suggesting the discriminative stimulus effects of α2/α3 subtype-selective GABA_A PAMs are weaker than nonselective GABA_A PAMs. Weaker discriminative stimulus effects can imply a lower risk of abuse potential than classical benzodiazepines.
CHAPTER 5- DISCUSSION AND CONCLUSIONS

Millions of individuals endure persistent pain. In the United States alone, about 30% of the population is estimated to suffer from chronic pain (Gilron et al., 2013). In addition to the physical and emotional burden that chronic pain imparts, chronic pain represents a financial challenge, costing the nation up to $635 billion in treatment and lost productivity in 2010 (Gaskin & Richard, 2012). Currently, the most widely used analgesics include μ-opioid agonists, anti-inflammatory steroids, and nonsteroidal anti-inflammatory drugs (NSAIDs). While these analgesics are useful in some pain conditions, their use is limited due to lack of efficacy and various side-effects. Thus, the development of novel and effective analgesics remains a clinical need.

Emerging evidence suggests that positive allosteric modulators that target GABA\(_A\) receptors could be a novel class of analgesics. Specifically, positive allosteric modulators that are selective for α2/α3 – containing GABA\(_A\) receptors. Subtype-selective GABA\(_A\) receptor PAMs demonstrate antinociceptive effects in models of acute and chronic pain (Di Lio et al., 2011; McKernan et al., 2000). Furthermore, these compounds display a reduced side-effect profile when compared to classical nonselective benzodiazepines (Knabl et al., 2008). However, majority of the literature involving subtype-selective GABA\(_A\) PAMs describe GABA\(_A\) PAMs that are selective for α2, α3, and α5 subtypes. Since α5 subtypes are associated with cognitive effects (selectivity at the α5 subtype may impair cognition), α2/α3 subtype-selective GABA\(_A\) PAMs are proposed to have a more desirable side-effect profile. At the start of this dissertation work, there were little to no subtype-selective PAMs that were truly selective for α2/α3-containing GABA\(_A\) receptors, thus many things had not been addressed regarding α2/α3-
subtype-selective GABA<sub>A</sub> PAMs as potential analgesics. Firstly, while α2/α3 subtype-selective GABA<sub>A</sub> receptors had been shown to produce anxiolytic effects (Atack, 2005; Poe et al., 2016) they had not been shown to demonstrate antinociceptive and analgesia effects in models of acute and chronic pain. Secondly, the potential use of α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs in combination therapy with currently available analgesics, such as opioids, had yet to be explored. Lastly, the pharmacological and behavioral profiles of α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs remained underexplored. Thus, the experiments conducted in this dissertation were aimed to provide insight on these underexplored areas and ultimately assess the potential of α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs as a novel class of analgesics.

The studies discussed in chapter 2A demonstrated that the degree to which α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs can attenuate acute pain is dependent on i) the model of acute pain and ii) the degree of pain. While α2/α3 subtype-selective GABA<sub>A</sub> receptor PAM KRM-II-81 failed to significantly increase latency in the tail-flick assay, KRM-II-81 attenuated pain in the acute visceral pain model by dose-dependently decreasing the number of writhes. The ability for KRM-II-81 to restore acid-depressed nesting (a pain-depressed behavior) was dependent on the degree of pain. KRM-II-81 restored 0.32% lactic acid-depressed nesting, but failed to restore 0.6% acetic acid-depressed nesting (a higher degree of pain, than lactic acid). In chronic pain models, α2/α3 subtype-selective GABA<sub>A</sub> receptor PAMs KRM-II-81 and NS16085 are effective for attenuating mechanical, but not thermal hyperalgesia (chapter 2B). Since, this was observed in both the neuropathic and inflammatory pain model, it is possible that α5-subtypes are necessary in order to attenuate thermal hyperalgesia. GABA<sub>A</sub> PAMs that are selective for α2/α3/α5 subtypes have been shown to mediate both thermal and mechanical hyperalgesia. Another explanation for KRM-II-81 and NS16085 only attenuating mechanical
hyperalgesia, is that different α-subtypes may be expressed differently among the different classes of nociceptors. It is possible that α2/α3-containing GABA$_A$ receptors are expressed largely in mechanical nociceptors (nociceptors responding to mechanical stimuli), and not expressed in thermal nociceptors (nociceptors responding to thermal stimuli) or polymodal nociceptors (nociceptors that respond to mechanical, chemical, and thermal stimuli).

Furthermore, the benzodiazepine site antagonist flumazenil attenuates the analgesic effects of GABA$_A$ PAMs (midazolam, KRM-II-81, and NS16085), confirming that the analgesic effects are mediated via the benzodiazepine site of the GABA$_A$ receptor. Lastly, studies discussed in chapter 2C demonstrate that α2/α3 subtype-selective GABA$_A$ receptor PAMs can produce robust analgesia devoid of tolerance development. While rats that received twice-daily injections of midazolam became tolerant to the analgesic effects of midazolam (after 7 consecutive days), tolerance was not observed in rats receiving daily injections of KRM-II-81 nor NS16085 (after 11 consecutive days) in both inflammatory and neuropathic pain models.

Collectively, these findings give insight on the analgesic potential of α2/α3 subtype-selective GABA$_A$ receptor PAMs. The data presented in chapter 2 suggests that the α5 subtype is not necessary to produce robust analgesia and confirm the notion that the α1 subtype of the GABA$_A$ receptors is most likely contributing to analgesic tolerance that is observed when classical benzodiazepines are repeatedly administered.

The studies in chapter 3 showed that α2/α3 subtype-selective GABA$_A$ receptor PAMs are suitable for combination therapy with commonly prescribed opioids. α2/α3 subtype-selective GABA$_A$ receptor PAMs KRM-II-81 and NS16085 produced mainly additive and some supra-additive effects when combined with oxycodone and morphine in CFA-treated rats (as demonstrated in the mechanical nociception assay). There were no infra-additive (antagonistic)
effects observed in any of the fixed ratios between the GABA\(\text{A}\) PAMs and opioids. Furthermore, while rate-suppressing effects were observed when midazolam was combined with either oxycodone or morphine, rate-suppressing effects were not observed when \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs were combined with either of the opioids. These findings suggest that \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs may be more suitable for combination therapy than nonselective benzodiazepines, such as midazolam. Collectively, these findings further support the notion that \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs are clinically effective, in that not only can these compounds manage pain alone, but when combined with opioids they are able to produce at least additive effects. Clinicians have been encouraged not to prescribe opioids and benzodiazepines together. The reason being, that studies have shown combining benzodiazepines and opioids can increase overdose risk (Kim, McCarthy, Hoppe, Mark Courtney, & Lambert, 2018). Combining opioids with \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs (instead of nonselective benzodiazepines) may reduce the high overdose risk associated with benzodiazepine-opioid combinations.

Lastly, the studies discussed in chapter 4A are particularly relevant to the clinic, in that classical benzodiazepines produce various pharmacological effects simultaneously. Many of these effects are undesirable, thus limiting their therapeutic potential. The primary finding in chapter 4 is that \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs can selectively produce analgesic effects. However, the nonselective benzodiazepine midazolam produced rate-suppressing, analgesia, cognitive impairment, and myorelaxant activity at similar doses. In addition, the studies in chapter 4B gave insight on the discriminative stimulus effects of \(\alpha2/\alpha3\) subtype-selective GABA\(\text{A}\) receptor PAMs. Reliable discrimination was not acquired in the group of rats that were being trained to discriminate KRM-II-81 from its vehicle. This suggests that the
discriminative stimulus effects of α2/α3 subtype-selective GABA_A receptor PAMs are weaker than nonselective GABA_A receptor PAMs, such as midazolam. Furthermore, the discriminative stimulus effects of midazolam (along with other classical benzodiazepines) is primarily mediated by α1-subtype GABA_A receptors. Partial substitution of KRM-II-81 and NS16085 for midazolam discrimination suggests these compounds have limited in vivo pharmacological activity at α1-subtype containing GABA_A receptors.

While KRM-II-81 and NS16085 are both α2/α3 subtype-selective GABA_A PAMs, there were some differences that were observed. Findings from chapter 4 suggest that NS16085 has a more desirable side-effect profile than KRM-II-81. Muscle relaxant activity was not observed in NS16085 up to 56 mg/kg, while KRM-II-81 produced significant myorelaxant activity at 17.8 mg/kg KRM-II-81, producing a therapeutic index of 5.98. In addition, in the drug discrimination study, we found that 100 mg/kg NS16085 did not significantly increase midazolam-associated lever responding, while 32 mg/kg of KRM-II-81 significantly increased midazolam-associated lever responding. Although, food-maintained operant responding was suppressed at these particular doses, it is also possible that NS16085 has weaker discriminative effects that KRM-II-81. Collectively, findings from these behavioral studies confirm the subtype-selectivity of KRM-II-81 and NS16085. Ultimately, supporting the notion that α2/α3 subtype-selective GABA_A receptor PAMs have a more desirable side-effect profile when compared to classical benzodiazepines, which produce an array of pharmacological effects all at similar doses.

Taken together, the studies discussed in this dissertation strongly support targeting α2/α3-containing GABA_A receptors for pain management. These novel compounds have the potential to be used as a monotherapy and/or in combination therapy with μ-opioid receptor agonists. While the studies discussed in this dissertation provide compelling evidence that α2/α3 subtype-
selective GABA_A receptor PAMs are effective as candidate analgesics, there are still concepts left to explore in regard to α2/α3 subtype-selective GABA_A receptor PAMs as potential analgesics. Firstly, studies investigating the long-term effects of α2/α3 subtype-selective GABA_A receptor PAMs are still needed. Although, the development of analgesic tolerance was studied, it is possible that other off-target effects are observed upon prolonged use of α2/α3 subtype-selective GABA_A PAMs. Secondly, while investigating the discriminative stimulus effects shed some light on the drug-abuse potential of these compounds; the reinforcing effects and withdrawal effects of α2/α3 subtype-selective GABA_A PAMs still need to be extensively studied. Nevertheless, the findings reported in this dissertation provide further evidence of the analgesic potential of α2/α3 subtype-selective GABA_A receptor PAMs.
REFERENCES


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