

**Endogenous Modulation of Addiction: Chronic Pain and the
NMDA/NOS Cascade**

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Carrie Lynn Wade

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ABSTRACT

Opioid treatment for chronic pain is controversial due to abuse potential and perceived addiction potential. Because of perceptions of addiction from chronic opioid treatment for pain it is important to clearly understand the biological bases for a number of factors related to opioid therapy in the context of chronic pain, including the effectiveness of opioid treatment under distinct conditions chronic pain and alterations in the effectiveness of opioid treatment under distinct conditions of chronic opioid pharmacotherapy. One way to approach this question is to study the changes that occur with chronic pain and see how those changes parallel those that occur with opioid addiction. Our approach to address the questions raised above is to apply a combination of rodent models of pain and opioid self-administration. In the first phase of this study we examine changes in oral fentanyl self-administration under distinct conditions of chronic pain including inflammatory pain, neuropathic pain and an idiopathic pain model of sickle cell anemia. The second set of studies examines the potential for an endogenous modulator of the NMDA/NOS cascade to interact with adverse opioid events such as tolerance and addiction. We observed that mice with inflammatory pain, neuropathic pain and sickle cell anemia had differential fentanyl self-administration profiles following induction of mechanical hyperalgesia. In the second set of studies we observed that agmatine reduced opioid-induced tolerance and abolished self-administration behaviors. We also found that endogenous agmatine may have a neuroprotective effect on these opioid effects.

TABLE OF CONTENTS

CHAPTER	PAGE
List of Figures	v
List of Tables	vi
I. Introduction	1
Applying Models of Opioid Self-Administration to Models of Chronic Pain....	6
Agmatine Effects on Antihyperalgesia and Inhibition of Opioid Self-administration.....	9
II. Fentanyl self-administration in chronic pain	
Title Page.....	13
Introduction.....	15
Methods and Materials.....	16
Results.....	20
Figures and Figure Legends.....	23
Discussion.....	32
III. Supraspinally administered agmatine attenuates the development of oral fentanyl self-administration	
Title Page.....	34
Introduction.....	36
Methods and Materials.....	37
Results.....	42
Figures and Figure Legends.....	47
Discussion.....	53
IV. Immunoneutralization of agmatine sensitizes mice to mu-opioid receptor tolerance	
Title Page.....	58
Introduction.....	60
Methods and Materials.....	62
Results.....	67
Figures and Figure Legends.....	74
Discussion.....	83

V. Chronic sequestration of endogenous agmatine inhibits opioid self-administration	
Title Page.....	87
Introduction.....	89
Methods and Materials.....	90
Results.....	90
Figures and Figure Legends.....	95
Discussion.....	97
VI. Summary and Conclusion.....	98
VII. References.....	104
VIII. Appendix.....	119

LIST OF FIGURES

CHAPTER	PAGE
II. Fentanyl self-administration in chronic pain	
Figure 1 A-D.....	23
Figure 2 A-D.....	25
Figure 3 A-C.....	27
Figure 4.....	28
Figure 5 A-D.....	29
Figure 6 A-D.....	31
III. Supraspinally administered agmatine attenuates the development of oral fentanyl self-administration	
Figure 1 A-F.....	47
Figure 2 A-C.....	48
Figure 3 A-C.....	49
Figure 4 A-C.....	50
Figure 5 A-C.....	52
IV. Immunoneutralization of agmatine sensitizes mice to mu-opioid receptor tolerance	
Figure 1 A-D.....	74
Figure 2.....	76
Figure 3 A-D.....	77
Figure 4.....	79
V. Chronic sequestration of endogenous agmatine in self-administration	
Figure 1 A-F.....	95
Figure 2.....	96

LIST OF TABLES

CHAPTER

PAGE

IV. Immunoneutralization of agmatine sensitizes mice to mu-opioid receptor tolerance

Table 1.....	81
Table 2.....	82

Chapter I

Introduction

The most pervasive and universal form of human distress is pain. It has an inverse relationship with one's quality of life (Weinstein et al. 2000; Weinstein et al. 2000) and negatively impacts collective social productivity (Blyth et al. 2003). Pain is one of the most common reasons for a visit to a physician's office, community health center and emergency rooms and is now considered the 5th vital sign. When pain interferes with and at times halts the activities of one's daily activities, pain becomes central to that person's daily existence. It is estimated that 50 million people experience chronic pain at some level in the United States, but that only 25% are provided adequate pain management (Davis 2003).

While most acute pain can be easily treated and is essential for disease diagnosis, conversion to a chronic pain condition often becomes more difficult to treat and retains little value in terms of identification of pending damage, dysfunction, or disease. Such chronic pain conditions become challenging to treat for a variety of reasons. First, chronic pain can arise from a wide variety of central or peripheral nervous system dysfunctions that are highly specific in origin (e.g. inflammation, neuropathy, disease-specific, cancer-related). In many cases the pain is caused by multiple points of dysfunction. Such conditions are often difficult to treat pharmacologically (Lynch et al. 2008; Scascighini and Sprott 2008; Scascighini et al. 2008) for reasons associated both with the neurobiology of pain and the chemistry of pharmacological agents used to treat pain. Specifically chronic pain of differing etiology may evoke differential

neurochemical and molecular changes at multiple levels of the central nervous system (Woolf and Decosterd 1999). These alterations often occur at the protein and specifically the receptor level. They may both affect targets used for pharmacological pain management and may present new as yet undercharacterized or unknown targets for pain management. At the level of chemistry, there currently are fairly limited options for pharmacological treatments for chronic pain. That is an area of universal disappointment for pain researchers, industry, practitioners, patients and their families.

The primary and most effective class of pharmacological agents used to manage chronic pain remains the opioid agonists. Opioid receptors are expressed at nearly every point in the pain signal conduction pathway (primary afferent peripheral nerve terminal, primary afferent central terminal, second order neurons, rostral ventral medulla, periaqueductal gray, and thalamus). Therefore they are well positioned to regulate the pain signal and, of the inhibitory GPCRs, they appear to be the most effective target for this purpose. Consequently, opioids have been used for centuries to control pain. Unfortunately, opioid receptors are also expressed in brain regions that drive addictive behavior. The experience of opioid agonists in human addictive behavior has also been described and well known for centuries. For many years the assertion was made that chronic pain patients who take opioid medication for treatment of their pain self-administer to attain the analgesic effect and rarely convert to become addicted (Chapman and Hill 1989). It was on that basis that prescribers have been increasingly educated and re-trained to appropriately treat patients for their pain, which is often undertreated. It has been asserted that only 25% percent of chronic pain patients receive adequate treatment (Davis et al.

2003). There has been increased education of practitioners as the appropriate use and prescribing protocols became a high priority and emphasis area. Concerns were raised regarding practitioner bias against prescribing opioids starting at the level of medical school students (Weinstein et al. 2000) and other health professionals such as pharmacists and regulators (Gilson and Joranson 2001). It was noteworthy that among medical board regulators the perceived legal and medical acceptability of treating patients with a history of opioid misuse, even for cancer-related pain, was significantly reduced relative to patients without a history of misuse (Gilson and Joranson et al., 2001). The introduction of sustained-release opioids to the pain management armamentarium in the 1990s provided extensive initial enthusiasm (Davis 2003) in terms of improved pharmacokinetics in that the new sustained-release formulations offered serum levels of opioids that could be maintained at steady-state for 12-24 hours. Such a pharmacokinetic profile meant that patients needing such chronic pain management would be at significantly less risk for breakthrough pain associated with opioid regimens reflecting shorter half lives that result in more frequent drops in serum levels that are below the minimal effective concentrations (Davis 2003). Therefore, initially, the introduction of sustained-release opioid medication was considered a significant advance in treatment. However, concern regarding opioid therapy for pain management is still controversial for at least two reasons: the continued perceived misuse of opioids and the lack of efficacy in non-malignant pain management.

The assertion has previously been made from limited sets of data that opioid use in subjects with neuropathic pain, for example, was not effective. Research investigating the

effectiveness of morphine in neuropathic pain patients showed that morphine (Arner and Meyerson 1988) was not effective in certain forms of neuropathic pain. However, this study was limited in scope in terms of patient population and dosing schedule. This study examined patients with severe neuropathic pain that had been unresponsive to nerve block, surgery, TENS (transcutaneous electrical nerve stimulation) and drug therapy including other opioids such as buprenorphine and pentazocine. The patients in this study received two doses of 10-20 mg morphine i.v. and were evaluated 15 minutes following infusion. This study highlights the importance of incorporating specific pain conditions, including inflammatory and neuropathic pain, in preclinical trials; it noted that most preclinical trials only tested drugs in nociceptive pain such as tail flick and hotplate. The overall conclusions have been challenged by many preclinical and clinical trials (Erichsen et al. 2005; LaBuda and Little 2005; Wu et al. 2005) and, yet, are still used to support the argument against use of opioids for neuropathic pain. La Buda et al. (2005) examined efficacy of several pharmacological agents that are used for treatment of neuropathic pain, Gabapentin, an $\alpha 2\delta$ antagonist and morphine reversed spinal nerve ligation-induced hyperalgesia with complete efficacy. Other drugs that are commonly used to treat neuropathic pain such as amitriptyline, a serotonin reuptake inhibitor, and indomethacin, a cyclooxygenase inhibitor, did not result in significant reversal of mechanical hyperalgesia (LaBuda and Little 2005). Several reports have contributed to the growing acceptance of the assertion that patients with non-cancer pain may achieve good pain control from opioid therapy (Kirsh et. al., 2002). However, there is also consensus that the current clinical literature on opioid use for the treatment of non-cancer

pain does not address issues of long-term opioid maintenance and that such studies are critical to inform management plans for treatment of non-cancer chronic pain patients with opioid therapy (Eisenberg et al., 2005; Foley, 2003).

It remains unclear whether there is a biological basis for restricting the use of opioids in patients with chronic pain, particularly of non-malignant origin. The National Institutes of Health has recently acknowledged this problem and has attempted to address this issue by developing research programs specifically to investigate the relationship of prescription opioid use and misuse, CNS changes that occur with chronic pain, and how these changes parallel those that occur with drug addiction. In 2005 and 2008 RFAs were announced that specifically addressed these issues with an emphasis on clinical research in the first case (2005 November 2005 RFA DA-06-005) *Prescription of Opioid Use and Abuse in the Treatment of Pain* (NIDA/NIA/NIDCR) and an emphasis on basic research in the second case (RFA-DA-09-017, October 2008) *Central Nervous System Intersections of Drug Addiction, Chronic Pain and Analgesia* (NIDA/NINDS).

While opioid research has traditionally focused on either addiction or chronic pain, it is of recent interest to apply behavioral models of both addiction and pain research to evaluate addiction in the context of chronic pain. Evidence using heroin as an analgesic opioid shows that rats with neuropathic pain do not, in fact, escalate opioid intake over the course of the self-administration period compared to their control counterparts (Martin et al. 2006; Martin et al. 2007; Martin and Ewan 2008). Using fentanyl as an analgesic opioid, Colpaert and colleagues have shown that rats with poly-arthritis, a model for chronic inflammatory pain, self-administer to an analgesic state but do not self-

administer opioids when pretreated with a non-opioid non-steroidal anti-inflammatory drug (NSAID), such as indomethacin (Colpaert et al. 2001). These results show that it is possible that rats in pain self-administer for the reward of analgesia alone and not for extra-analgesic reward.

Merging behavioral models for chronic pain and self-administration is an important aspect of this research. It is also important to elucidate the physiological mechanisms that may protect against or exacerbate the addiction potential of commonly used opioids when taken for chronic pain conditions. The processes of addiction and the onset of chronic pain share at least one similar physiological pathway, that of the NMDA receptor/nitric oxide synthase (NOS) cascade. Activation of this system has been shown to play a crucial role in the induction of many chronic pain states (Liu and Sandkuhler 1998; Sandkuhler 2007). Blocking this cascade in the spinal cord prevents the onset of many neuropathic and inflammatory pain states. Similarly, many NMDA receptor antagonists have been shown to play a role in addiction (Morgan et al. 2002; Wolf et al. 2003; Nugent et al. 2007).

Applying Models of Opioid Self-Administration to Models of Chronic Pain

Most rodent studies of opioid anti-hyperalgesic neuropharmacology in neuropathic pain make measurements acutely at one time point during the progression of the pain state. However, the most valuable information to be gained from models may require evaluation of opioid pharmacotherapy over a significant period of time. The mouse models of neuropathic pain and inflammatory hyperalgesia present an opportunity to make controlled comparisons of the complexity of opioid pharmacotherapy in terms of

opioid sensitivity or insensitivity, development of tolerance (controlling for progression of disease), and factors pre-disposing to or protecting from addiction.

Much has been learned about the basic biological and environmental factors that influence addiction in animal models using the self-administration paradigm (Campbell and Carroll 2000). The self-administration paradigm is used as a sensitive measure of a drug's rewarding effects, and the potential addiction liability of drugs can be inferred from the results. Generally, drugs that are addictive in humans are readily self-administered by laboratory animals such as amphetamines, cocaine, heroin, and cannabinoids (Bergman and Paronis 2006; Sanchis-Segura and Spanagel 2006; Panlilio and Goldberg 2007). Several groups have shown that mice with pain will self-administer opioids differently than normal controls (Lyness et al. 1989; Colpaert et al. 2001; Martin et al. 2007; Martin and Ewan 2008). It has been shown that self-administration of fentanyl can be correlated with the amount of spontaneous pain associated with different pain conditions, monoarthritis and mononeuropathy (Kupers and Gybels 1995; Backonja and Gosnell 1996). Similarly, the examination of the self-administration behaviors of rats experiencing chronic nociceptive pain in an arthritis model shows that rats with CFA-induced polyarthritis self-administer fentanyl for analgesic effects based on a decrease in self-administration rates when given forced fentanyl IV (Colpaert et al. 2001). When given a choice between two bottles of water, one with fentanyl and one with water, mice with arthritis consumed more fentanyl water than the non-pain control. When only fentanyl water was available, non-arthritic rats consumed more fentanyl overall (Colpaert et al. 2001).

Yet another study demonstrates a reduction in IV self-administration of morphine in rats with CFA-evoked polyarthritis compared to pain free control rats. Tail pressure evoked nociception in rats self-administering morphine and indomethacin, a non-opioid anti-inflammatory also show a decreased sensitivity. There was also a reduction in opioid self-administration in the rats that received indomethacin, a potent NSAID (Lyness et al. 1989). To date, no study following the time course of escalating pain state compared with escalation of opioid intake has been done. This research program significantly expanded upon the concepts of the previous studies (Lyness et al. 1989; Kupers and Gybels 1995; Backonja and Gosnell 1996; Colpaert et al. 2001) to evaluate fentanyl self-administration in a neuropathic model of hyperalgesia and to compare self-administration motivated by ongoing pain to that motivated only by drug reward.

The research program presented within this thesis had the intention to develop an optimally controlled experimental environment to directly compare chronic opioid self-administration under conditions of inflammatory, neuropathic, and idiopathic pain. The program was designed to evaluate opioid self-administration in this condition over time considering disease progression. CFA has been shown to produce hyperalgesia of nine day duration (Ren and Dubner 1996; Djouhri et al. 2001). Because this hyperalgesia is short-term compared to the time course of approximately 14-21 days for neuropathic pain (Honore et al. 2006) the comparison of fentanyl intake between the two groups was expected to provide information about motivation for fentanyl intake, tolerance and addiction. Finally, we can compare pain in mice that is idiopathic in nature by using the disease-specific model of sickle-cell anemia (Fabry et al. 1995).

Agmatine Effects on Antihyperalgesia and Inhibition of Opioid Self-administration

Endogenous glutamate and NMDA receptors play a critical role in spinal nociceptive processing (Davar et al. 1991; Kristensen et al. 1992; Mao et al. 1992; Yamamoto and Yaksh 1992; King and Lopez-Garcia 1993; Malmberg and Yaksh 1993; Meller et al. 1993; Meller and Gebhart 1993; Ren and Dubner 1993; Sluka and Westlund 1993; Mathisen et al. 1995; Nikolajsen et al. 1996; Pud et al. 1998; Svendsen et al. 1998; Wong et al. 1998), the induction of spinal opioid tolerance (Dunbar and Yaksh 1996; Fairbanks and Wilcox 1997), and the acquisition of opioid self-administration behavior (Semenova et al. 1999; Xi and Stein 2002). The downstream mediator of NMDAR activation, nitric oxide, has also been implicated in many of these processes (Kitto et al. 1992; Babey et al. 1994; Huang et al. 1994; Fin et al. 1995; Good 1996; Hamada et al. 1996; Collins and Kantak 2002). Evidence reported in the early 1990s invoked hypotheses correlating spinal opioid tolerance with spinally mediated hyperalgesia, suggesting that they shared common mechanisms of induction mediated by glutamate, NMDAR and protein kinase C (Chen and Huang 1992; Mao et al. 1994; Mao et al. 1995; Mao et al. 1995). In fact, NMDAR antagonists and NOS inhibitors prevent adaptive changes in neuronal function, including long-term potentiation (Haley et al. 1992), opioid tolerance (Trujillo and Akil 1991; Fairbanks and Wilcox 1997) and dependence (Aricioglu-Kartal and Uzbay 1997; Li et al. 1999), persistent pain (Chaplan et al. 1997; Yoon et al. 1998; Fairbanks et al. 2000), and spinal cord injury (Faden and Simon 1988; Faden et al. 1989; Wu and Li 1993; Wu et al. 1994; Yu et al. 2000).

Agmatine is an amine and organic cation formed by the decarboxylation of L-arginine by the enzyme arginine decarboxylase in bacteria, plants and invertebrates (Tabor and Tabor 1984). It was discovered in mammals in the mid 1990s (Li et al. 1994; Raasch et al. 1995) where it was found to be expressed, among other organs, in the CNS. Agmatine is constitutively present in brain (Otake et al. 1998; Reis and Regunathan 1998) and spinal cord tissue (Fairbanks et al. 2000) suggesting that it is present throughout the CNS. That its synthetic (Li et al. 1994) and degradative (Iyer et al. 2002; Mistry et al. 2002) enzymes are thought to also be present in CNS makes it likely that endogenous agmatine serves a modulatory role in CNS function. In addition, agmatine antagonizes the NMDA receptor (Yang and Reis 1999; Fairbanks et al. 2000; Roberts et al. 2005; Wade et al. 2009) and inhibits the activity of all isoforms of NOS (Auguet et al. 1995; Galea et al. 1996; Fairbanks et al. 2000; Roberts et al. 2005; Wade et al. 2009). That it antagonizes NMDA receptors and inhibits NOS positions agmatine mechanistically as an endogenous modifier of neuroadaptive changes such as neuropathic pain and acquisition of opioid self-administration behavior.

Studies from our research group constituted the first evidence that spinally delivered agmatine altered established CNS processes in vivo: intrathecally administered agmatine delivered concomitantly with tolerance-inducing doses of morphine prevented the development of acutely induced tolerance (Fairbanks and Wilcox 1997); and intrathecally delivered agmatine overcame sensitization of paw withdrawal responses of mice following inflammation or nerve injury (Fairbanks et al. 2000). Others (Horvath et al. 1999) had previously shown that pre-treatment with agmatine attenuated the development

and maintenance of inflammation-evoked thermal hyperalgesia, but the results presented by Fairbanks and colleagues (Fairbanks and Wilcox 2000) constituted the first demonstration of reversal of established sensitization by post-treatment. Specifically, a single injection of a low dose of agmatine one day following induction of hyperalgesia by dynorphin or spinal nerve injury reversed the hyperalgesia, apparently permanently. Spinally delivered agmatine also dose-dependently inhibits both NMDA-evoked scratching and biting behavior as well as NMDA-evoked thermal hyperalgesia (Fairbanks et al. 2000), results which are consistent with electrophysiological and biochemical evidence describing its activity at NMDA receptors and NOS (Kitto et al. 1992).

Agmatine has also been demonstrated to effectively decrease the escalation of i.v. fentanyl self-administration in rats (Morgan et al. 2002). The escalation of drug intake in animals has been proposed to be a critical indicator of the transition from controlled drug use to drug addiction in humans (Ahmed and Koob 1998). Using a transition model of addiction, multiple systemic doses of agmatine effectively attenuated the increase in fentanyl self-administration occurring over successive days of drug exposure (Morgan et al. 2002). The attenuating effects of agmatine on fentanyl self-administration are specific to the initial phases of the transition from moderate to excessive intake. This indicates a potential modulatory role of agmatine in the behavioral phenomenon of drug self-administration. This observation suggests that agmatine can moderate neuroadaptive events related to long-term opioid self-administration and may serve as a potentially useful clinical pharmacotherapy. The initial focus of this research program was evaluation of opioid self-administration in chronic pain models. We then evaluated the

role of an endogenous modulator of the NMDA glutamatergic system in our model of fentanyl self-administration; the participation of NMDA/NOS cascade has been well established in processes of neuronal adaptation, including both chronic pain and opioid addiction and represents a clear potential common mechanism between the two phenomena.

Chapter II

Fentanyl self-administration in chronic pain

The goal of this study was to evaluate fentanyl self-administration under varying conditions of chronic pain. We tested the following hypothesis: chronic pain will alter fentanyl self-administration.

The following people contributed to this work:

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(C.L.W. planned and conducted the experiments. B.W. characterized sickle cell anemia hyperalgesia profile and conducted experiments. D.S. conducted experiments, P.K. conducted experiments. C.A.F. assisted with method development, interpretation and editing.)

It is important to clearly understand the biological basis for a number of factors related to opioid pharmacotherapy for chronic pain, including, but not limited to, the following: 1. The effectiveness of opioid treatment under distinct conditions of chronic pain. 2. Alterations in the effectiveness of opioid treatment under distinct conditions of chronic opioid pharmacotherapy. Our approach to address the questions raised above was to apply a combination of rodent models of pain and opioid self-administration. In the first set of studies oral fentanyl self-administration was assessed in ICR mice trained to lever press for orally delivered fentanyl (70 μ L) in daily 2 hour sessions (3-4 weeks) in mouse models of 1) chronic inflammatory pain (CFA), 2) neuropathic pain and 3) a disease specific model of sickle cell anemia. Mice with CFA-induced inflammatory pain, spinal nerve ligation- and chemotherapy-induced neuropathic pain and an idiopathic pain type induced by sickle cell anemia had differential fentanyl self-administration profiles following induction of mechanical hyperalgesia. In the second set of studies under the previous conditions mice were trained to self-administer food in daily two hour sessions (2 weeks). Food-maintained responding was not altered in the chronic pain models relative to respective control levers. These studies indicate that fentanyl self-administration can discretely measure differences in opioid preference under several chronic pain conditions.

Introduction

Opioid efficacy has been well established in several chronic pain conditions including inflammation, neuropathic pain and disease states, such as cancer and sickle cell anemia. Patient-controlled analgesics using i.v. morphine or fentanyl formulations such as lollipops and transdermal patches are often employed for pain management, but are controversial because of perceptions of overuse and addiction liability. Several groups have studied the reward potential of opioids under conditions of nerve-injury and inflammation using physiological techniques (Berhow et al. 1996; Nestler et al. 1996; Ozaki et al. 2004) and behavioral assays (Ozaki et al. 2004; Niikura et al. 2008; King et al. 2009). Extracellular signal-related kinase (ERK) activity in the reward centers of the brain has been examined under conditions of non-contingent chronic morphine administration with and without chronic pain (Lyness et al. 1989; Berhow et al. 1996; Colpaert et al. 2001; Ozaki et al. 2004; Martin et al. 2006; Martin et al. 2007; Martin and Ewan 2008). Berhow et al., 1996 showed that ERK activity increased in the ventral tegmental area (VTA) following implantation of a morphine pellet and this ERK activity subsequently increased tyrosine hydroxylase activity, which is a biomarker for dopamine production in the reward centers. Blocking ERK activity through antisense targeted towards ERK blocked the increase in tyrosine hydroxylase production (Berhow et al. 1996). Ozaki et al., 2004 examined this phenomenon under the condition of neuropathic pain and showed that rats with spinal nerve ligation (SNL)-induced neuropathic pain fail to show a place preference paired to morphine injection, which is often used as a measure of reward (Tzschentke 1998). In a separate experiment, conditioned place preference was

also inhibited in a dose-dependant manner as a result of i.c.v. injection of a specific MEK inhibitor, PD98059, which blocks ERK activity. Ozaki et al. (2004) also demonstrated that rats with neuropathic pain have decreased ERK activity in the VTA compared to their sham control counterparts. These results indicate that under the condition of neuropathic pain, morphine may not have the same rewarding properties as when an animal is pain free.

The physiological data showing decreased activity in reward centers following induction of neuropathic pain indicates that it is likely that opioids lose at least some rewarding effects under conditions of chronic pain, but it is not known if this translates to altered opioid intake when an animal is allowed to have free access to an opioid. Several groups have explored this question and have shown there are in fact differences in the consumption of opioids under a variety of pain conditions (see introduction for review) (Lyness et al. 1989; Colpaert et al. 2001; Martin et al. 2006; Martin et al. 2007; Martin and Ewan 2008). Using an operant conditioning model of self-administration it is possible to examine differences in escalation and maintenance between mice in chronic pain states for the duration and offset of pain. We have developed a model of fentanyl self-administration to evaluate opioid intake during several chronic pain conditions, including pain of inflammatory and neuropathic origin and a model of idiopathic pain using sickle cell anemia.

Materials and Methods

Animals. Experimental subjects were either Institute of Cancer Research (ICR) male mice (21-30 g, Harlan, Madison) or C57BL mice (see sickle cell anemia subheading).

Subjects were housed in groups of eight in a temperature- and humidity-controlled environment and maintained on a 12 hr light/dark cycle with free access to food and water. These experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Chemicals. Quinine hydrochloride, CFA and vincristine sulfate were purchased from Sigma Chemical (St. Louis, MO). Fentanyl citrate was purchased from Gallipot (St. Paul, MN). CFA and vincristine sulfate were mixed in 0.9% NaCl. Fentanyl citrate and quinine hydrochloride were dissolved in distilled water.

Self-Administration apparatus. Experimental chambers were Modular Mouse Test Chambers (Med-Associates, ENV-307CT, St. Albans, VT). Each chamber was housed in a sound-attenuating cubicle (Med-Associates, ENV-021M), and equipped with a 3.33 RPM syringe pump (Med-Associates, PHM-100) for drug delivery, 20 mg food pellet delivery system (Med-Associates, ENV-203-20), 2 ultra sensitive mouse levers (Med-Associates, ENV-310M) and 2 stimulus lights (Med-Associates, ENV-321M). A 4.8 W house light located at the top of the cage was illuminated during experimental sessions.

Behavioral procedure. The FR1 reward schedule coupled an active lever press with a delivery of 70 μ l drug solution to the receptacle, and illumination of the stimulus light directly above the lever. After each reward, there was a 5 second time-out period during which no reward was possible, regardless of additional lever presses (which will also be recorded). Responding on the control lever resulted only in illumination of the stimulus light above it. Animals in the non-fentanyl control groups received dH₂O (+ quinine) instead of fentanyl, which controlled for the possibility that motivation for fluid (rather

than drug) is the reinforcer. Responses were monitored for both the active lever (the lever that drives delivery of fluid) and the control lever (the lever for which there was no associated reward provided in response to being pushed) and expressed as mean responses for each test day. The control lever controlled for random activity in the operant chamber during which that lever may be pushed. Each mouse was tested once daily (2 hour session) for the duration of the experiment.

CFA-induced hyperalgesia. Mice were injected with 30 μ l of a 50% solution of CFA the day before the first self-administration session. Mice were tested for mechanical hyperalgesia using von Frey filaments before and after the second session and every 3 or 4 days throughout the duration of the experiment.

Spinal nerve ligation-induced neuropathic pain. Hypersensitivity was induced by surgical ligation of the L5 spinal nerve in mice. Mice were anesthetized with isoflurane and a mini-Goldstein retractor (Fine Science Tools No. 17002-02, Foster City, CA) with a 1-cm maximum spread was then inserted into the incision at the level of the iliac crest to expose the L6 transverse process and the rostral tip of the sacrum. The L6 transverse process was then removed with use of an S&T fine forceps (Fine Science Tools No. 00108-11). Removal of the process permits visual identification of the L4–L5 spinal nerves. The L5 spinal nerve was tightly tied (ligated) with 6-0 silk thread distal to the dorsal root ganglion and proximal to the confluence of spinal nerves L4 and L5. The animals were fully mobile within 30 min of cessation of anesthetic. As a control, in a separate group of animals, a sham surgery identical to the aforementioned procedure (but

without nerve ligation) was performed. Mice were tested for mechanical hyperalgesia using von Frey filaments throughout the duration of the experiment.

Chemotherapy-induced neuropathic pain. In the first study mice were injected with either saline, 0.03, 0.1 or 0.3 mg/kg of vincristine sulfate, i.p. once daily for 10 days and mice were assessed for mechanical hyperalgesia using an electronic von Frey anesthesiometer (IITC Life Science) for a period of 14 days. In a separate study vincristine sulfate (0.1 mg/kg) was injected i.p. the day of the first self-administration session and for 9 days following the first injection. Hyperalgesia was confirmed using electronic von Frey assessment throughout the duration of the experiment.

Sickle cell anemia. Mice used were inbred C57BL control mice, transgenic mice expressing human α - β^s -globin transgenes with a C57BL background with a natural deletion of the murine β -globin gene, transgenic control mice (HbA-BERK) expressing human α - and β -globin transgenes, with homozygous knockout of the murine α - β -globin genes, and transgenic sickle mice (hBERK) expressing human α - β^s -globin transgenes, with a homozygous knockout of the murine α -globin gene and a heterozygous of the murine β -globin gene. Subjects were housed in groups of four in a temperature- and humidity-controlled environment, maintained on a 12h light/dark cycle. Water and food were given *ad libitum*. During the fentanyl self-administration period mice were given water *ad libitum*, but fed on a restricted diet of 3 grams per day throughout the duration of the experiment. These experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Data Analysis. The area under the curve (AUC) was determined by the trapezoidal rule

using the statistical software package JMP[®] 6 from SAS. The resulting AUCs were analyzed using analysis of variance (ANOVA) from Prism 4.0. Significance was defined as $P < 0.05$.

Results

CFA-induced inflammatory pain. Figure 1 shows that mice with CFA-induced hyperalgesia (A) do not have a significant difference in lever pressing between the active and control lever for the time course that mechanical hyperalgesia was present (phase 1) as assessed by the area under the curve (C). When hyperalgesia returned to baseline by day 15 (phase 2), mice with CFA-induced hyperalgesia had an increase in lever pressing for fentanyl and they did discriminate between the active and control lever as assessed by the area under the curve (D). The lack of discrimination between the active and control levers that was observed in phase 1 was not reproduced in food-maintained responding indicating that mobility was not the primary reason for decreased lever pressing (figure 3A).

Neuropathic pain. Mice with spinal nerve ligation (SNL)-induced neuropathic pain responded less for fentanyl compared to the sham and naïve controls following acquisition of the self-administration behavior (figure 2A). We further evaluated fentanyl self-administration from day 17-24 and saw that the control mice increased their fentanyl responding at a level greater than the mice with neuropathic pain (see figure 2 insets). Figure 2D represents an area under the curve for days 17-24 and shows that the difference between the active lever and control lever is significant for both control groups whereas responding on the active lever is not significant compared to the control lever for

the ligated mice. Hyperalgesia was confirmed for the duration of the study. The decrease in lever pressing was not reproduced in food-maintained responding indicating that mobility was not the primary reason for decreased lever pressing (figure 3B).

We also examined self-administration in a less invasive model of neuropathic pain. We employed a chemotherapy drug-induced model of neuropathic pain. The benefit of this model is that neuropathic pain can be achieved with daily intraperitoneal (i.p.) injections and does not require invasive manipulations. We first characterized mechanical hyperalgesia in a dose response curve and found that 0.1 mg/kg resulted in the most reliable hyperalgesia (figure 4) and that mice receiving this dose did not show any signs of other sickness behaviors, such as decreased grooming and weight loss (observations). We observed that hyperalgesia was achieved by day 4 and was returning to baseline by day 14. In a separate experiment, in which we used this dose of vincristine sulfate, we tested mice in fentanyl self-administration for a period of 20 days with daily injections from day 1-14. Hyperalgesia was evident by day 4 and continued for the duration of the experiment. It is evident that mice with vincristine-induced neuropathic pain (figure 5A) did not self-administer fentanyl during the time that they were hyperalgesic as compared to their saline-injected counterparts (figure 5B). Figure 5C is a representation of the area under the curve for the duration of the experiment.

Sickle cell anemia. The primary objective of the overall study was to evaluate phenotypic changes in hyperalgesia associated with sickle cell anemia (SCA). The hyperalgesia assays we used to assess the sickle cell mice did not show any differences between the SCA mice and their control counterparts (Willis 2007). We tested the mice

in a model of fentanyl self-administration to assess if this model could show more subtle differences between the SCA mice and their controls. Using a concentration of 10 $\mu\text{g/ml}$ of fentanyl (+ 30 $\mu\text{g/ml}$ quinine) sickle cell anemia mice had increased fentanyl responses compared to the control group. After 23 days of fentanyl intake mice had a 23-day extinction period where water (+ 30 $\mu\text{g/ml}$ quinine) was substituted for fentanyl. Over this time period SCA mice decreased their response rate to match the control group, indicating the mice were responding specifically for fentanyl (figure 6A and B). When we tested the mice in food-maintained responding we found that the SCA mice responded less than the control group excluding the possibility that the mice were more active in their self-administration chambers (figure 6C and D).

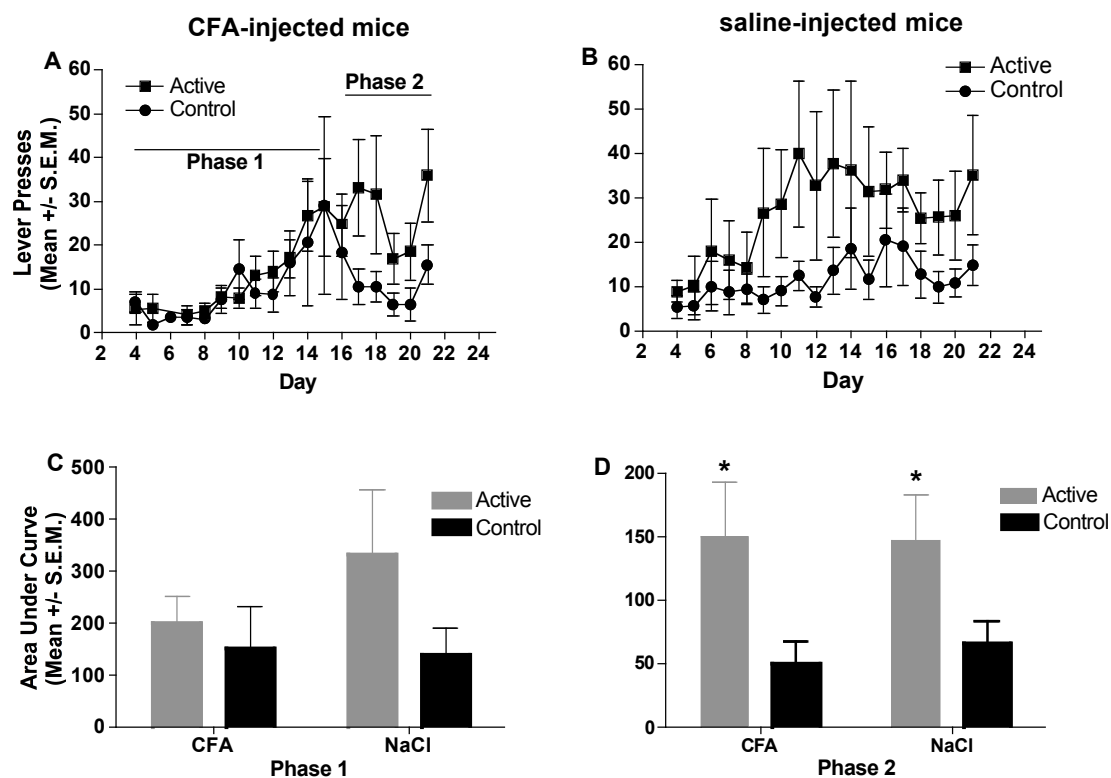


Figure 1. Fentanyl self-administration in CFA-induced inflammation: Time course of fentanyl self-administration for mice with CFA-induced inflammatory pain (A) saline-injected control (B). Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/ml) (squares). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Analysis of the AUC for the groups in A and B show that during phase 1 (C), when mice were hyperalgesic, animals that had CFA-induced inflammation had reduced lever pressing as

compared to saline-injected controls, and that in phase 2, when mice were no longer hyperalgesic, they had a significant increase in fentanyl lever pressing. Complete Freund's Adjuvant was injected intraplantar the day before the first self-administration session. Mechanical hyperalgesia was assessed for 12 days before and after self-administration sessions and was reduced by day 12 and returned to baseline by day 15 (data not shown). (*significance was determined by ANOVA; $p < 0.05$). N=8 per group.

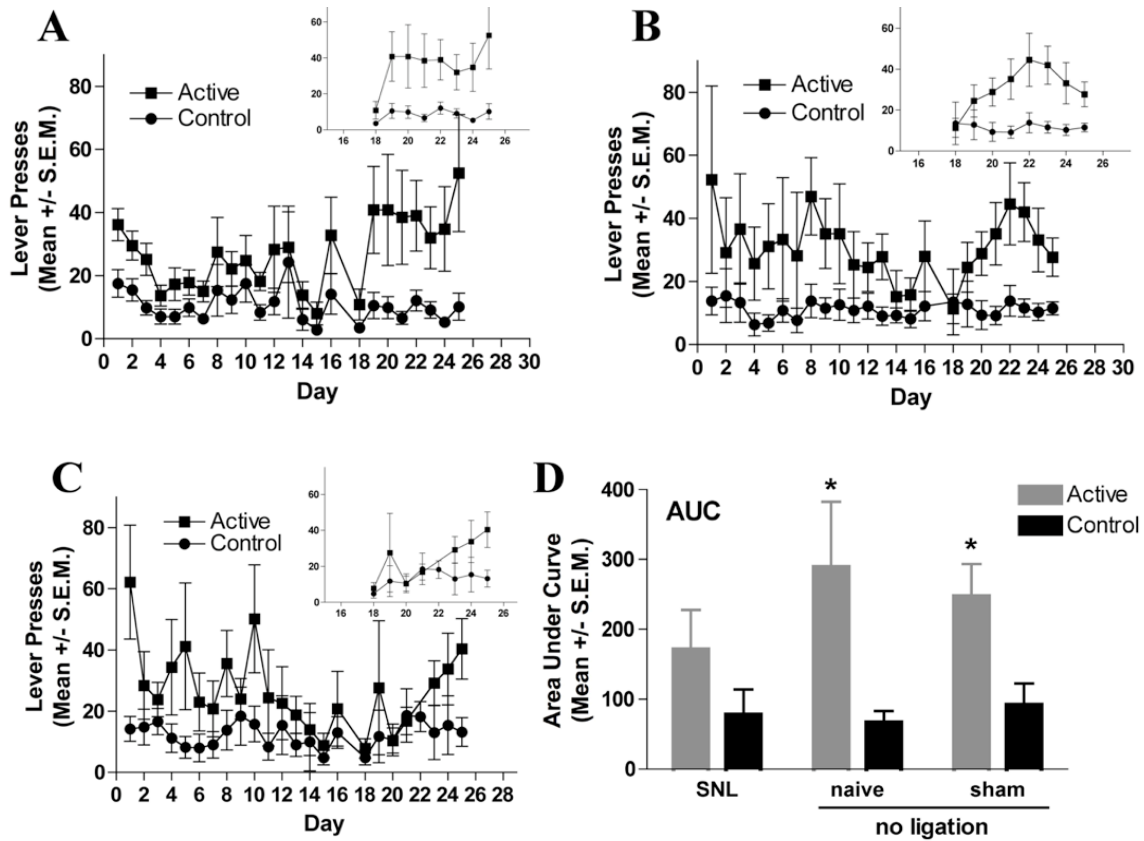


Figure 2. Fentanyl self-administration in spinal nerve ligation-induced neuropathic pain: Time course of fentanyl self-administration for naive (A) and sham control (B) and mice with SNL-induced neuropathic pain (C). Spinal nerve ligation and sham control surgery was done 2 days before the first self-administration session. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/ml) (squares). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Analysis of the AUC (D) for the groups in A-C show that

animals that had SNL-induced neuropathic pain did not lever press for fentanyl suggesting that neuropathic pain inhibited the development of fentanyl self-administration behavior. (*significance was determined by ANOVA; $p < 0.05$). N=7 per group

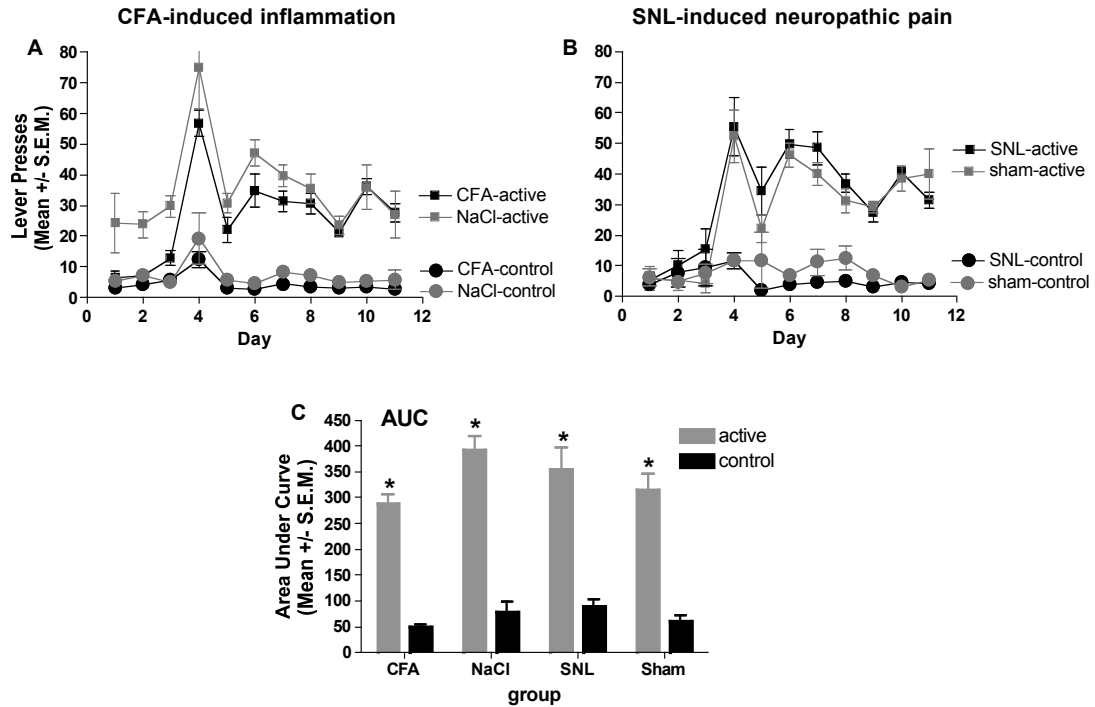


Figure 3. Food-maintained responding in CFA-induced inflammatory and SNL-neuropathic pain: CFA was injected intraplantarly the day before the first food self-administration session and spinal nerve ligation surgery was done 2 days before the first food self-administration session. Food responding did not change for CFA-induced inflammatory pain (A) or for SNL-induced neuropathic pain (B). Food-maintained responding and control lever presses were recorded in daily 2hr sessions. (*indicates significant difference in responding between the respective control and active lever within experimental group was determined by ANOVA, $p < 0.05$). $N=6$ per group

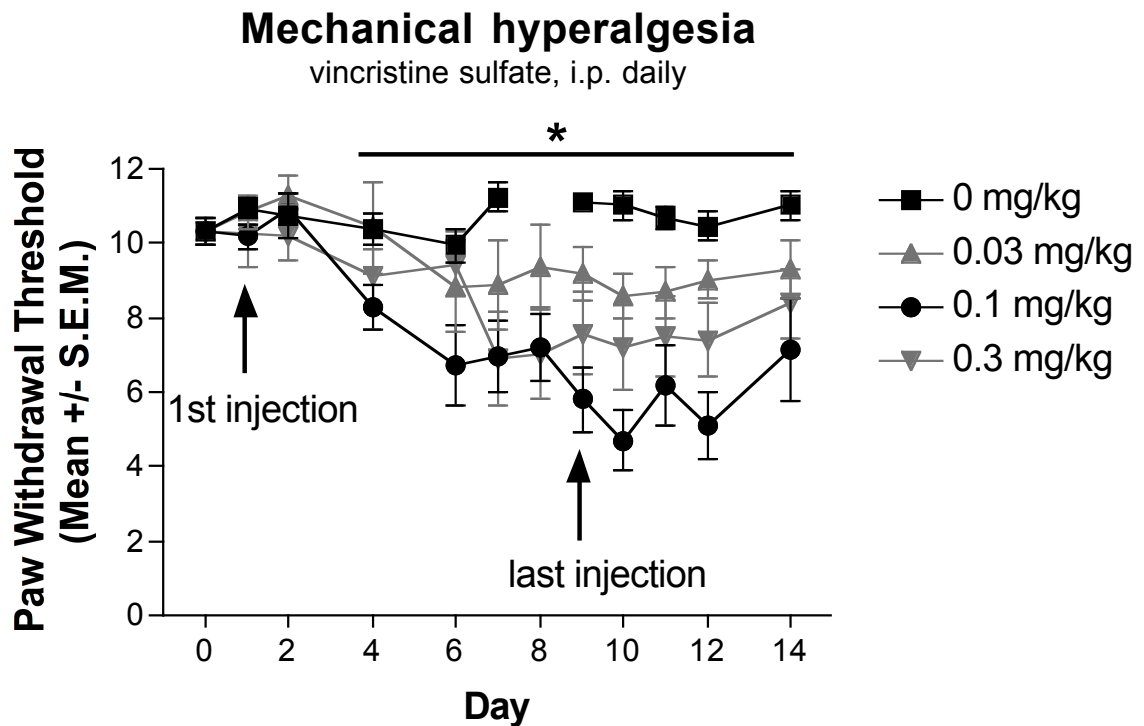


Figure 4. Chemotherapeutic-induced neuropathic pain: Vincristine was injected daily for 9 days at different doses (0.03, 0.1, 0.3 mg/kg, i.p.) following an initial baseline. Mechanical hyperalgesia was assessed using an electronic vonFrey anesthesiometer for 14 days. (*indicates significant difference in mechanical hyperalgesia between the respective saline- and 0.1 mg/kg vincristine- injected animals, i.p. was determined by ANOVA, $p < 0.05$). N=8 per group

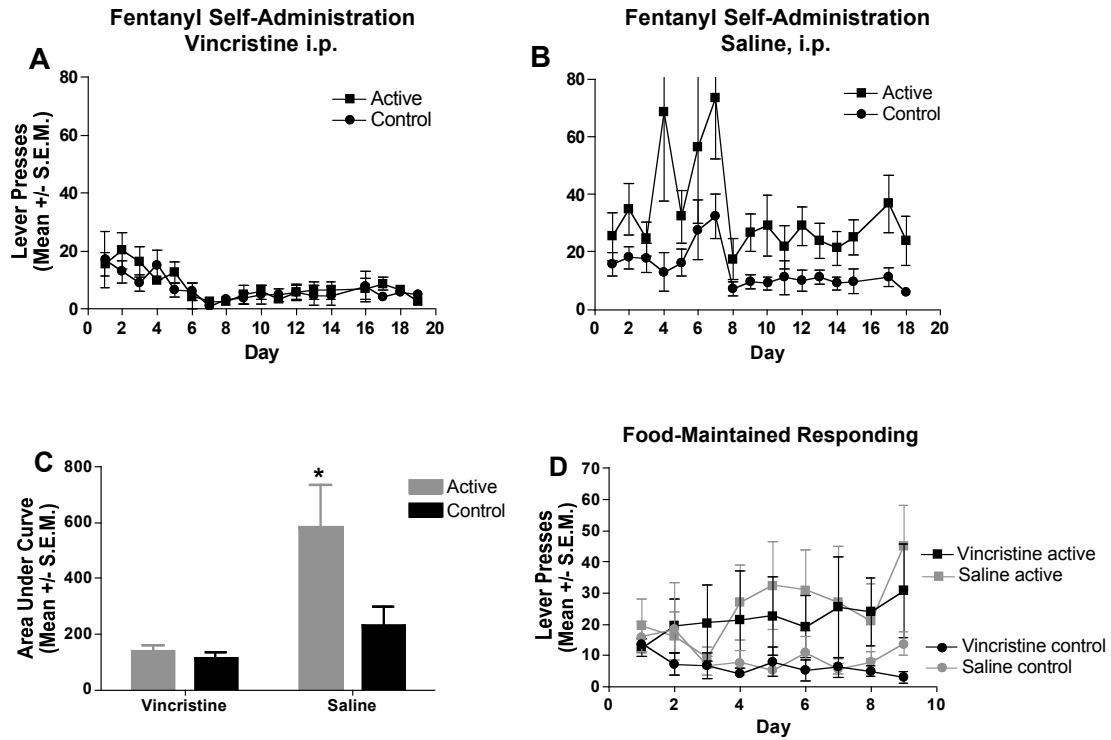


Figure 5. Fentanyl self-administration in vincristine-induced neuropathic pain:

Time course of fentanyl self-administration for mice receiving 0.1 mg/kg (i.p.) vincristine sulfate (A) or 0.9% saline (B) the day of the first self-administration session and once daily for 14 days. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/ml) (squares). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Analysis of the AUC (C) for the groups in A and B show that animals that had vincristine-induced neuropathic pain did not lever press for fentanyl suggesting that neuropathic pain inhibited the development of fentanyl self-administration behavior. In a different

experiment food-maintained responding was measured in both groups (D). There was no difference in responding indicating that mice did not have decreased fentanyl self-administration due to mobility issues. (*significance was determined by ANOVA; $p < 0.05$).

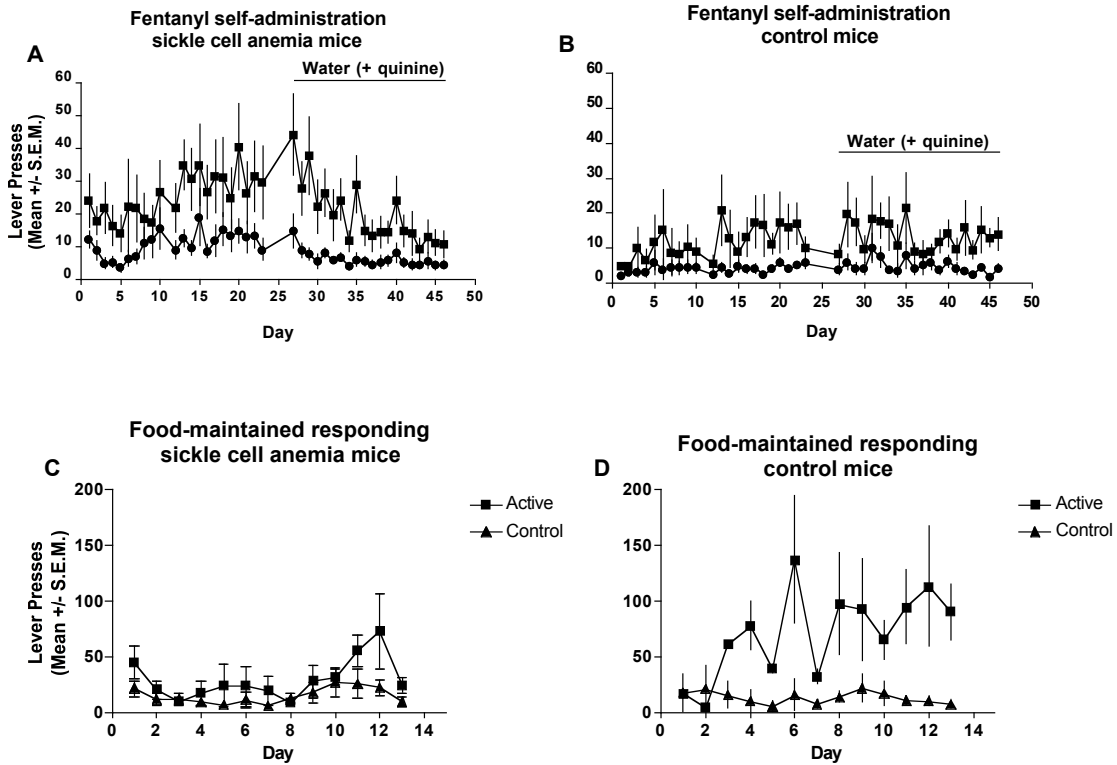


Figure 6. Fentanyl self-administration in an idiopathic pain model: Time course of fentanyl self-administration for mice with sickle cell anemia (A) and controls (B). Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/ml) (squares). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Food responding was decreased for SCA mice (C) compared to the heterozygous control group (D). Food-maintained responding and control lever presses were recorded in daily 2hr sessions. N=8 per group.

Discussion

To study the effect that chronic pain states have on the abuse potential of opioids we chose to employ an operant conditioning paradigm where mice were able to freely lever press for the desired amount of fentanyl. This allows us to examine possible motivation and limits of opioid intake. We studied three forms of chronic pain that are clinically relevant for administration of opioid therapy and found that all three forms of chronic pain resulted in differences in fentanyl intake. Mice with inflammatory and neuropathic pain responded less for fentanyl than their control counterparts and mice with a disease-specific pain state (SCA) responded more for fentanyl.

To evaluate fentanyl self-administration in a model of neuropathic pain that did not require surgery we employed a different model of non-invasive chemotherapy-induced neuropathic pain. This model requires only a single i.p. injection daily for 10-14 days and produces robust hyperalgesia. In fact, we found that mice with vincristine-induced neuropathic pain did not self-administer fentanyl during the time they were hyperalgesic. These data lead to the possibility that under the condition of neuropathic pain, opioids are less rewarding.

We started this research program by evaluation of inflammatory pain which has been well studied in preclinical models and well treated in clinical settings and then evaluated a more difficult pain state, that of neuropathic pain using spinal nerve ligation- and chemotherapy-induced hyperalgesia. Finally, we studied phenotypic changes in a disease model that results in an idiopathic pain type. It has been well established that sickle-cell anemia patients describe chronic non-specific pain as well as severe breakthrough pain

described as sickle-cell crisis (Ballas 2010; Wilkie et al. 2010). Although this is well described, the model of sickle-cell anemia that we used produced no obvious phenotypic changes in pain behavior or morphine efficacy. We used fentanyl self-administration to evaluate possible differences in fentanyl preference and found that in fact SCA mice responded more for fentanyl than their control counterparts. This preference seemed to be specific for fentanyl in that they had decreased responding when the fentanyl solution was switched for H₂O (+30 µg/ml quinine). This appears to be the first experiment of its kind.

Together these data suggest that opioids lose at least some of their rewarding properties when taken in the context of chronic pain. Opioid self-administration has previously been studied in SNL-induced neuropathic pain by Martin and colleagues (2007). Two main findings from this study were that rats with neuropathic pain had differential profiles of opioid self-administration; with every opioid they tested, rats had increased lever pressing with doses of opioids that were higher than those preferred with control rats. They also showed that rats with neuropathic pain had decreased lever pressing for opioid when they were pretreated with non-opioid analgesics such as clonidine, suggesting that rats were self-administering for the reward of pain relief. Our findings from inflammatory- and neuropathic-induced pain support this conclusion.

Reasons for the loss of reward associated with opioids in chronic pain conditions are not fully understood, but could be a result of decreased dopamine production in the reward centers of the brain, specifically the VTA (Narita et al. 2004; Ozaki et al. 2004). Behavioral data suggest this may be the case.

Chapter III

Supraspinally administered agmatine attenuates the development of oral fentanyl self-administration

This study focuses on the development of the oral fentanyl self-administration model and the effects of agmatine on the development of fentanyl self-administration. The first objective was to develop a working model of oral fentanyl self-administration that is clinically relevant. Our second objective was to evaluate the role of the NMDA/NOS cascade in the acquisition and maintenance of fentanyl self-administration. We tested the following hypothesis: agmatine given centrally attenuates opioid self-administration.

The following data was published:

Wade CL, Schuster DJ, Domingo KM, Kitto KF, Fairbanks CA. Supraspinally Administered Agmatine Attenuates the Development of Oral Fentanyl Self-Administration. *European Journal of Pharmacology*. 587 (2008) 135-140.

(C.L.W. planned and conducted the experiments and wrote the paper. D.J.S., K.M.D., and K.F.K. conducted some experiments. C.A.F. assisted with method development, interpretation and editing.)

The decarboxylation product of arginine, agmatine, has effectively reduced or prevented opioid-induced tolerance and dependence when given either systemically (intraperitoneally or subcutaneously) or centrally (intrathecally or intracerebroventricularly). Systemically administered agmatine also reduces the escalation phase of intravenous fentanyl self-administration in rats. The present study assessed whether centrally (intracerebroventricular, i.c.v.) delivered agmatine could prevent the development of fentanyl self-administration in mice. Mice were trained to respond under a fixed-ratio 1 (FR1) schedule for either fentanyl (0.7 μ g/70 μ l, p.o.) or food reinforcement. Agmatine (10 nmol/5 μ l), injected i.c.v. 12-14h before the first session and every other evening (12-14h before session) for 2 weeks, completely attenuated oral fentanyl self-administration (but not food-maintained responding) compared to saline-injected controls. When agmatine was administered after fentanyl self-administration had been established (day 8) it had no attenuating effects on bar pressing. This dose of agmatine does not decrease locomotor activity as assessed by rotarod. The present findings significantly extend the previous observation that agmatine prevents opioid-maintained behavior to a chronic model of oral fentanyl self-administration as well as identifying a supraspinal site of action for agmatine inhibition of drug addiction.

Introduction

The organic cation agmatine (decarboxylated L-arginine) was first identified in the mammalian central nervous system (CNS) in 1994 (Li et al. 1994). Since then, we and others have confirmed its presence in CNS through immunohistochemistry and other bioanalytical methods such as high pressure liquid chromatography (HPLC) and mass spectrometry. Agmatine meets many of the criteria of a central neuromodulator. Agmatine is synthesized, stored, and released from specific networks of neurons, is inactivated by energy-dependent reuptake mechanisms and is enzymatically degraded (Reis and Regunathan 1998). The presence of its synthetic (Li et al. 1994) and degradative (Iyer et al. 2002; Mistry et al. 2002) enzymes in CNS supports the proposal that endogenous agmatine serves a modulatory role in CNS function. In addition, we have recently also shown that [³H]-agmatine is transported into and released from synaptosomes (purified nerve terminals)(Goracke-Postle et al. 2006) under conditions of K⁺ stimulation in a Ca²⁺-dependent manner (Goracke-Postle et al. 2007; Goracke-Postle et al. 2007).

Agmatine is unique in that it both antagonizes N-methyl D-aspartate (NMDA) receptors (Yang and Reis 1999; Fairbanks and Wilcox 2000) and inhibits (Auguet et al. 1995; Galea et al. 1996) or inactivates (Demady et al. 2001) nitric oxide synthase (NOS), a property that distinguishes it from most exogenously applied NMDA receptor antagonists and NOS inhibitors, which usually act at just one site. Taken together with the role of NMDA receptors and NOS in neuroplasticity (Haley et al. 1992), these actions suggest that agmatine may serve as an endogenous modulator of glutamatergic neuroplasticity.

When given exogenously, agmatine inhibits several requisite processes in glutamatergic neuroplasticity: it is neuroprotective in models of spinal cord injury and brain ischemia (Gilad and Gilad 1996), permanently interrupts neuropathic pain, and blocks opioid tolerance and dependence. It was also found that agmatine, given by repeated intravenous (i.v.) infusions, reduces escalation of intravenous fentanyl self-administration (Morgan et al. 2002). When given i.v. *ter in die* (t.i.d.) during the self-administration session, agmatine attenuated (but did not completely ablate) the escalation phase of i.v. fentanyl self-administration, indicating that agmatine's effects extend beyond CNS adaptation to chronic opioid treatment and includes opioid-driven reward. Despite agmatine's short (<10 min) plasma half-life (Raasch et al. 2002; Piletz et al. 2003; Roberts et al. 2005), its systemic administration consistently affects a wide variety of CNS-mediated processes (Nguyen et al. 2003). Based on the 12h CNS half-life reported by our group (Roberts et al., 2005; (Chu et al. 2007), we hypothesized that intermittent i.c.v. administration would yield prolonged activity; in fact, agmatine given once daily or once every other day completely prevented the development of supraspinal opioid-induced tolerance (Kitto and Fairbanks 2006). In order to determine whether agmatine could similarly exert a complete inhibition of fentanyl-self administration, the present study evaluated the effects of i.c.v.-administered agmatine in a mouse model of oral fentanyl self-administration.

Materials and Methods

Animals. Experimental subjects were Institute of Cancer Research (ICR) male mice (21-24 g, Harlan, Madison). Subjects were housed in groups of eight in a temperature- and humidity-controlled environment, maintained on a 12h light/dark cycle. Water was given

ad libitum and mice were fed on a restricted diet of 3 grams per day throughout the duration of all experiments. Each mouse was used in only one experimental group. These experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Chemicals. Agmatine sulfate was purchased from Sigma Chemical (St. Louis, MO) and dissolved in 0.9% saline. Fentanyl citrate was purchased from Gallipot (St. Paul, MN) and dissolved in distilled water (dH₂O). Quinine hydrochloride (Sigma) (30 µg/ml) was included in both the fentanyl and the control water to reduce the potential for taste preferences for one fluid over the other. This approach has been used in other studies of oral fentanyl self-administration (Kupers and Gybels 1995; Colpaert et al. 2001).

Intracerebroventricular injection. All drug- or saline-treated controls were administered i.c.v. in a 5 µl volume in conscious mice according to the method of Haley and McCormick (Haley 1957). All injections were performed by one experimenter (KFK) who has over fifteen years of experience with the procedure.

The procedure for the injection was as follows: conscious mice were covered with a cloth to expose just the top of the head. The subject was then restrained at the base of the skull with the experimenter's thumb and forefinger so that the neck and jaw of the mouse were firmly, but gently, pressed against a firm flat level surface. A 50 µl Hamilton syringe was fitted to a 27-gauge needle with a rubber stopper positioned to expose 1.5 mm of the needle tip. The exposed tip was then inserted into the right lateral cerebral ventricle, through the scalp and the skull, 1 mm to the right of the skull's midline and level with the external auditory meatus; the skull was sufficiently soft to permit this insertion with

minimal force. Once the needle was positioned, 5 μ l of solution was injected and the needle removed. This procedure takes less than a minute and requires no anesthetic, surgery, or incision.

Self-Administration apparatus. Experimental chambers were Modular Mouse Test Chambers (Med-Associates, ENV-307CT, St. Albans, VT). Each chamber was housed in a sound-attenuating cubicle (Med-Associates, ENV-021M), and equipped with a 3.33 RPM syringe pump (Med-Associates, PHM-100) for drug delivery, 20 mg food pellet delivery system (Med-Associates, ENV-203-20), 2 ultra sensitive mouse levers (Med-Associates, ENV-310M) and 2 stimulus lights (Med-Associates, ENV-321M). A 4.8 W house light located at the top of the cage was illuminated during experimental sessions.

Behavioral procedure. The FR1 reward schedule coupled an active lever press with a delivery of 70 μ l drug solution to the receptacle, and illumination of the stimulus light directly above the lever. After each reward, there was a 5 second time-out period during which no reward was possible, regardless of additional lever presses (which will also be recorded). Responding on the control lever resulted only in illumination of the stimulus light above it. Animals in the non-fentanyl control groups received dH₂O (+ quinine) instead of fentanyl, which controlled for the possibility that motivation for fluid (rather than drug) is the reinforcer. Responses were monitored for both the active lever (the lever that drives delivery of fluid) and the control lever (the lever for which there was no associated reward provided in response to being pushed) and expressed as mean responses for each test day. The control lever controlled for random activity in the

operant chamber during which that lever may be pushed. Each mouse was tested once daily (2 hour session) for the duration of the experiment (24 days).

Experimental Designs. *Experiment 1:* The objective of this experiment was to evaluate the characteristics of oral fentanyl self-administration. There were two levers in the operant chamber, one that is an active lever the pressing of which resulted in delivery of 70 μ l of either fentanyl or water (the reward). The second lever was a control lever the pressing of which resulted in no reward. Pressing of both levers was tracked to determine the subjects' discrimination between the two levers. This schedule of reinforcement was applied to varying concentrations of oral fentanyl from 1-300 mg/ml and control, which was dH₂O (+ 30 mg/ml of quinine).

Experiment 2: The purpose of this experiment was to determine the effects of supraspinal agmatine on oral fentanyl self-administration using the optimal concentrations from experiment 1. The behavioral design was the same as described above, but 10 nmol/5ml of agmatine was delivered i.c.v. 12-15 hours before the first self-administration session (the evening before) and every 2 days for 16 days (for a total of 8 injections). Lower doses of agmatine (0.1 and 1 nmol) were also tested.

Experiment 3: The purpose of this experiment was to determine the effects of supraspinal agmatine on food-maintained responding using the optimal agmatine i.c.v. dose from experiment 2. To evaluate the effects of agmatine on food self-administration a separate group of mice was injected i.c.v. with agmatine the day before the first self-administration session and every 2 days for 16 days and followed the same behavioral

design, however, these mice were not exposed to fentanyl and only bar pressed for food pellets.

Experiment 4: The purpose of this experiment was to determine the effects of supraspinal agmatine on oral fentanyl self-administration after self-administration had been established. While the behavioral design was the same as described in Experiment 2, agmatine was administered after a minimum of 8 mice per group (agmatine vs. saline) had established fentanyl self-administration. The same schedule of i.c.v. injection was followed of agmatine administered every 2 days, starting on Day 8, after self-administration had been established. The criteria for inclusion were the following: the mice had to 1) respond for the fentanyl lever at a ratio of 2 or higher when compared to the control lever and 2) respond for the fentanyl at a minimum of 10 lever presses per session for 3 days in a row.

Experiment 5: (Note: This experiment is an addendum in that it was not included in the original manuscript, but belongs within the context of this specific line of investigation.)

The purpose of this experiment was to determine the effects of supraspinal agmatine on a reinstatement model of oral fentanyl self-administration. The behavioral design of the experiment was the same as described in experiment 2, but no i.c.v. agmatine was given. Mice responded for fentanyl for a period of 19 days and on day 20 mice went through a 30 day extinction period where water (+30 μ g/ml quinine) was substituted for the fentanyl solution. On day 47 mice were reinstated to fentanyl self-administration and either i.c.v. agmatine or saline was delivered every other day for 8 injections total. Self-administration was tracked throughout the experiment.

Rotarod assay. Following 2 consecutive training sessions, mice walk for 300 seconds on a rotarod apparatus (Accelerating Rotarod for Mice Ugo Basile Biological Research Apparatus, Varese, Italy) during which time the rotarod undergoes a linear acceleration from 4 to 40 rpm. We compared the latency to fall before and after administration of saline or agmatine.

Data analysis. The area under the curve (AUC) was determined by the trapezoidal rule using the statistical software package JMP[®] 6 from SAS. The resulting AUCs were analyzed using analysis of variance (ANOVA) from Prism 4.0. Significance was defined as $P < 0.05$.

Results

Oral Fentanyl Self-Administration. The primary objective of the overall study was to evaluate the impact of supraspinal agmatine treatment on oral self-administration of fentanyl. In order to pursue that aim, a protocol for establishing oral self-administration of fentanyl was needed. An experimental protocol specifically for the study of oral fentanyl intake in mice using an operant conditioning method has not been previously described. Therefore, we first evaluated a range of concentrations of fentanyl to determine the optimal concentration for establishment of a possible taste aversion using 30 mg/ml of quinine, we tested concentrations of fentanyl from 1, 10, 30 and 300 mg/ml and the vehicle, dH₂O (Fig. 1A-E).

Lever pressing for the vehicle control (dH₂O + 30 mg/ml quinine) did not differ between the active and control levers throughout the duration of the experiment (Fig. 1A). Lever pressing on the active lever for delivery of 1 and 10 µg/ml of fentanyl increased across

days 8-19 with the maximum mean reaching 50 active lever presses on day 19 (Fig. 1B,C). In this group there was no increase in responding on the control lever; the difference in response on the active and control levers was significant indicating a preference of lever pressing for drug delivery. A concentration of 30 mg/ml of fentanyl showed a maximum mean of 20 fentanyl active lever presses later in the experiment, but was not significantly different from that of the control lever (Fig. 1D) so discrimination between the levers was not evident. Finally, at the 300 mg/ml concentration of fentanyl mice showed minimal responding and no preference for fentanyl intake between the control and active levers over the duration of the study (Fig. 1E). Analysis of the AUC across the concentration range indicated that 10 mg/ml concentration of fentanyl was the highest concentration of fentanyl that showed statistically significant separation between the active and control levers with the least variability indicating the best representation of motivated self-administration behavior (Fig. 1F).

Agmatine attenuates oral fentanyl self-administration. We next evaluated the effect of supraspinally administered agmatine (i.c.v) on oral self-administration of 10 mg/ml of fentanyl. Agmatine (10 nmol) or saline was injected i.c.v. every other day until day 16 as described in the methods. Mice that received saline injections developed increased active lever pressing for fentanyl over time distinct from that of control lever pressing, as expected (Fig. 2A); the injection protocol did not impact the acquisition of the self-administration behavior. In contrast, agmatine treatment completely inhibited the responding of mice on the active lever, which did not differ from that of control levers for the duration of the experiment well beyond cessation of the drug delivery protocol (Fig.

2B). Delivery of two lower agmatine doses (1 and 0.1 nmol) also completely inhibited fentanyl self-administration (data not shown).

Agmatine does not affect food maintained-responding. We next examined the effects of i.c.v. agmatine (10 nmol) on food-maintained responding to evaluate the possibility that supraspinal agmatine treatment inhibited lever pressing in a non-specific manner. In contrast to the fentanyl self-administration experiment, agmatine pretreatment had no effect on the acquisition of food-maintained responding; mice responded for food pellet delivery comparably both in mice treated with supraspinal saline (Fig. 3A) or agmatine (Fig. 3B) with significant discrimination between the active and control levers in both treatment groups. This result argues against the possibility that the agmatine inhibition of fentanyl self-administration is a result or reduction of either learning or motor ability to perform the dependent measure. Consistent with that assertion, the ability of mice to stay on an accelerating rotarod device did not differ between mice pre-treated i.c.v. with saline (saline-treated: 238 ± 12 s) or agmatine (agmatine-treated: 245 ± 13 s). That these same doses did not impair lever presses in food-restricted mice or rotarod performance illustrate that the agmatine does not have an impact on satiety or motor function, excluding such effects as possible explanations for the reduction in lever presses in Fig. 2B.

Agmatine post-treatment does not impact fentanyl self-administration. In this experiment mice were trained to self-administer prior to the first agmatine injection. Those that reliably self-administered fentanyl by Day 8 were divided into two groups: 9 mice receiving 0.9% NaCl and 9 mice receiving 10 nmol/5 μ l agmatine i.c.v. In order to

be included mice had to respond for fentanyl at a ratio of 2 or greater compared to control lever presses and lever press for fentanyl a minimum of 10 times per session for 3 days; 75% of the mice met the criteria. Agmatine or saline was administered 12-14h prior to the ninth day of sessions and every other day thereafter as in the previous experiments. Mice that received saline injections maintained active lever pressing for fentanyl over time distinct from that of control lever pressing, as expected (Fig. 4A). As in Experiments 2 and 3, the i.c.v. injection schedule did not impact the acquisition of the self-administration behavior. However, in contrast to the result in Fig. 2B, agmatine-treatment after established fentanyl responding did not affect the responding of mice on the active lever; in other words, the agmatine-treated mice continued to discriminate between the active and control levers for the duration of the experiment (Fig. 4B). Therefore, this experiment shows that supraspinal agmatine treatment, when given after fentanyl self-administration was established, has no effect on fentanyl self-administration in contrast to the inhibitory effect of pre-treatment and continued treatment of agmatine during the acquisition phase of fentanyl self-administration (Fig. 2).

Agmatine treatment impacts reinstatement of fentanyl self-administration. *(Note: These data are an addendum in that they were not included in the original manuscript, but belong within the context of this specific line of investigation.)*

We evaluated the effect of supraspinally administered agmatine (i.c.v) on reinstatement of oral fentanyl self-administration. Following escalation of self-administration, mice had a 27-day extinction period where they were only exposed to water (+30 µg/ml quinine). After extinction, agmatine (10 nmol) or saline was injected i.c.v. every other day until

day as described in the methods. During the reinstatement period mice that received agmatine i.c.v. did not reinstate fentanyl self-administration (figure 5A) whereas mice that received saline injections had rates of fentanyl self-administration above that of the initial self-administration period (Figure 5B).

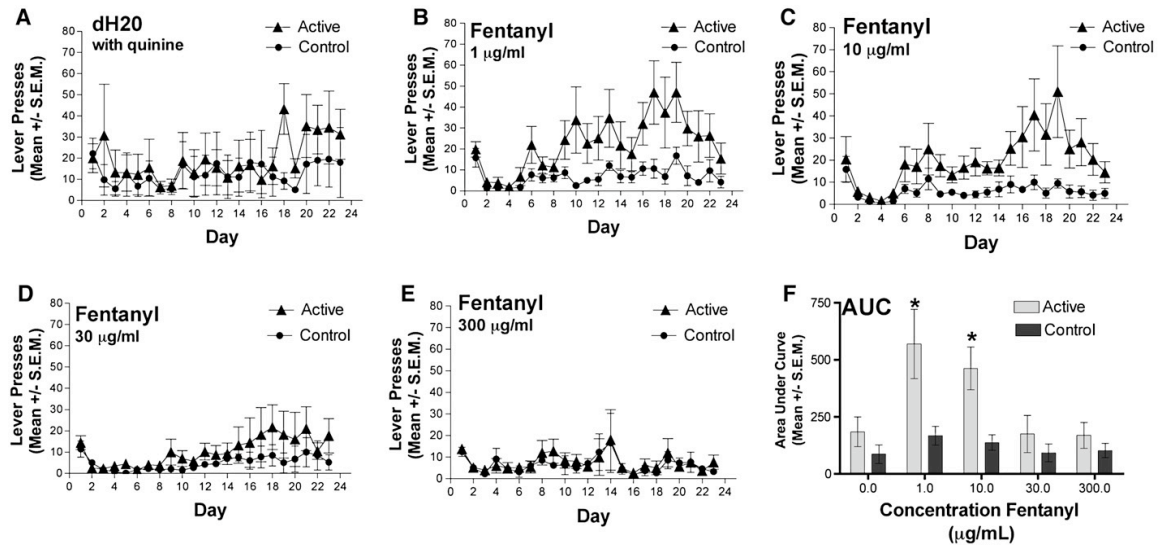


FIG. 1. Oral fentanyl self-administration. Mice did not respond for vehicle (dH₂O + 30mg/ml quinine (A)). Fentanyl self-administration peaked on Days 17-19 for 1 μ g/ml and Day 19 for 10 μ g/ml but concentrations (B and C) 30 μ g/ml and 300 μ g/ml failed to induce opioid self-administration (D and E). Data shown is representative of mean lever presses for the fentanyl response bar (active lever) (triangles; FR1) and the control lever (circles). Analysis of the area under the curve (AUC) across all self-administration sessions indicated that 10 mg/ml of fentanyl (+ 30 μ g/ml quinine) was the highest concentration of fentanyl that established self-administration behavior, showing statistically significant separation between the active and control levers (F) (*ANOVA $p < 0.05$). N=8 mice per group.

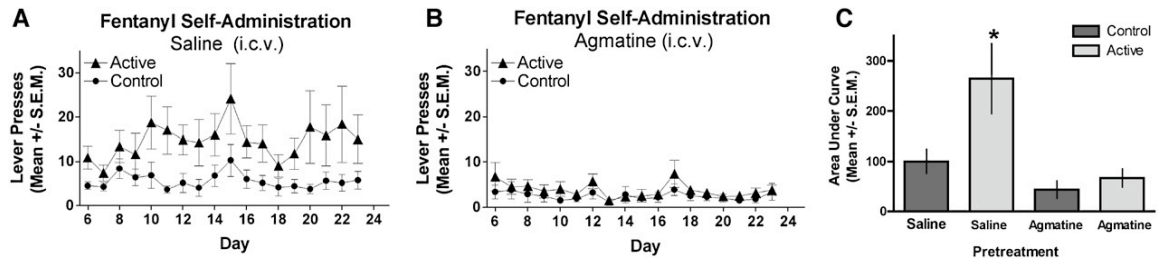


FIG. 2. Agmatine-mediated attenuation of fentanyl self-administration. Time course of fentanyl self-administration for mice receiving i.c.v. treatments of saline (A) or agmatine (10 nmol/ 5 μ L) (B) given the day before the first self-administration sessions and every 2 days for 16 days. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/ml) (triangles). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Mice did not respond differently for food-maintained responding under either the saline or agmatine conditions (C). Analysis of the AUC for the groups in A and B show that animals that received repeated i.c.v. saline discriminated between the control (1st bar, left to right) and the active (2nd bar) levers whereas the mice that received repeated agmatine did not discriminate between control (3rd bar) and active (4th bar) levers suggesting that agmatine inhibited the development of fentanyl self-administration behavior. (*significance was determined by ANOVA, $F_{(3,26)}$; $p < 0.05$).

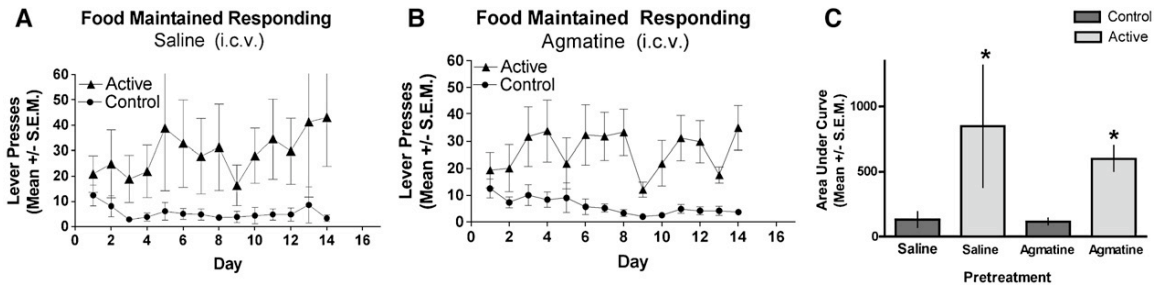


FIG. 3. Agmatine does not affect food-maintained responding. Time course of food-maintained responding for mice receiving i.c.v. treatments of saline (A) or agmatine (10 nmol/ 5 μ L) (B) given the day before the first self-administration sessions and every 2 days for 16 days. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 1 pellet of food (triangles). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Mice did not respond differently for food-maintained responding in either the saline (A) or agmatine (B) group indicating that agmatine has no effect on food reward. (C) Analysis of the AUC for the groups in A and B show that animals that received repeated i.c.v. saline discriminated between the control (1st bar, left to right) and the active (2nd bar) levers. The mice that received repeated agmatine also discriminated between control (3rd bar) and active (4th bar) levers suggesting that agmatine does not inhibit the response for food reward (*indicates significant difference in responding between the respective control and active lever within experimental group was determined by ANOVA, $F_{(3,28)}$; $p < 0.05$).

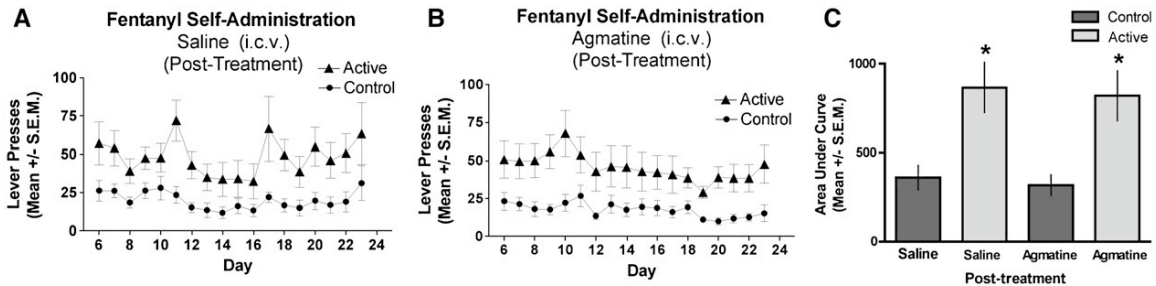


FIG. 4. Delivery of agmatine after the establishment of fentanyl self-administration.

Time course of fentanyl self-administration for mice receiving i.c.v. treatments of saline (A) or agmatine (10 nmol/ 5 μ L) (B) given after fentanyl self-administration has been established on day 8 and every 2 days for 14 days. The arrow on the graph denotes the first injection. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/mL) (triangles). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Only mice meeting inclusion criteria were tested (ratio of 2 or greater compared to control lever presses and that the mice had to lever press for fentanyl a minimum of 10 times per session for 3 days; 75% of mice met this criteria). Mice did not alter active lever responding in response to agmatine i.c.v. injections. (C) Analysis of the AUC for the groups in A and B show that animals that received repeated ICV saline continued to discriminate between the control (1st bar, left to right) and the active (2nd bar) levers. The mice that received repeated agmatine after establishment of self-administration behavior also continued to discriminate between control (3rd bar) and active (4th bar) levers suggesting that this

injection schedule of agmatine does not reverse the maintenance of fentanyl self-administration (*signifies difference in responding between the respective control and active lever within experimental group ANOVA, $F_{(3,32)}$; $p < 0.05$). N=9 mice per group.

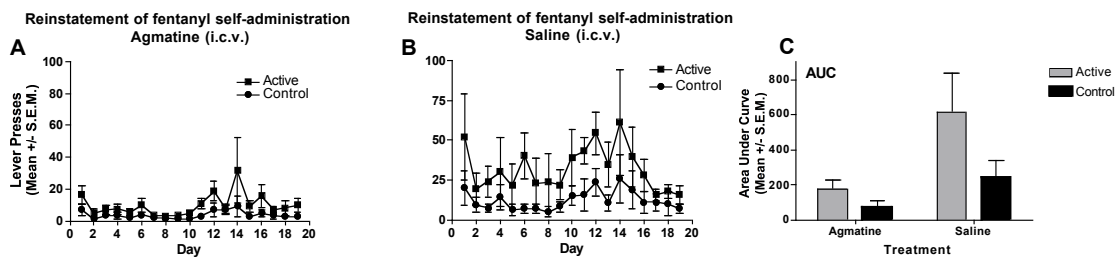


FIG. 5. Agmatine effects in reinstatement of fentanyl self-administration.

Time course of reinstatement of fentanyl self-administration for mice receiving i.c.v. treatments of agmatine (10 nmol/ 5 μ L) (A) or saline (B). After fentanyl self-administration was established mice were given water instead of fentanyl for 27 days to extinguish fentanyl responding. Following the extinguishing period, agmatine was given i.c.v. every 2 days the day before the first reinstatement session. Responses represent lever presses on one of two bars. The first bar (active lever) delivers 70 μ L of fentanyl (10 μ g/mL) (squares). Pressing the control lever results in no reward and is indicative of non-specific activity (circles). Although the data does not reach the level of significance with $p < 0.05$, there is a trend that mice did not lever press in response to agmatine i.c.v. injections compared to saline controls. (C) Analysis of the AUC for the groups in A and B. N=8 mice per group.

Discussion

The present study demonstrates that supraspinally administered (i.c.v.) agmatine prevents the acquisition and reinstatement of fentanyl self-administration. First, mice readily self-administered oral fentanyl at 10 $\mu\text{g/ml}$. Using that concentration of fentanyl it was observed that agmatine (0.1-10 nmol, i.c.v.) pretreatment completely inhibited fentanyl self-administration and decreased reinstatement of self-administration. That i.c.v. agmatine did not alter food-maintained responding or rotarod performance indicates that agmatine's action is specific to drug-reinforcing behavior and does not affect the ability of the mice to acquire the lever-pressing response. Further, the observation that agmatine (10 nmol, i.c.v.) post-treatment after fentanyl self-administration had been established had no effect suggests the importance of timing of the delivery of agmatine in the acquisition phase of the behavior. One other report has shown that the NMDA receptor antagonist, memantine, decreased reinstatement of opioid-conditioned place preference (Popik et al. 2006).

It has been previously shown that agmatine, whether given systemically (Kolesnikov, 1996) or centrally, prevents morphine tolerance (Kolesnikov et al. 1996; Fairbanks and Wilcox 1997; Kitto and Fairbanks 2006) and dependence (Aricioglu-Kartal and Uzbay 1997; Aricioglu et al. 2003; Aricioglu et al. 2004). Morgan and colleagues (Morgan et al. 2002) demonstrated that systemically administered agmatine (i.v.) reduced the escalation of fentanyl (but not cocaine) self-administration (i.v.). While this study did not specifically evaluate agmatine against cocaine self-administration, together, these data suggest that the relationship of agmatine may be specific to opioidergic systems. Further,

prior evidence has linked agmatine to the glutamatergic system, specifically as an NMDA receptor antagonist (Yang and Reis 1999) and NOS inhibitor (Galea et al. 1996; Demady et al. 2001). Such linkage is significant since NMDA receptor antagonism and NOS inhibition are both well established mechanisms for inhibition of opioid-evoked analgesic tolerance.

The role of NMDA receptor antagonists and NOS inhibitors in opioid self-administration studies has been less widely evaluated and is less clear. Evidence to support a role for the NMDA receptor includes the observation that Lewis rats with significantly higher NMDA receptor levels in specific brain regions reach higher breaking points (e.g. the progressive ratio where rats will respond more for a single infusion) and response ratios when compared to Fischer rats (Martin et al. 2003). Semenova and colleagues (Semenova et al. 1999), however, demonstrated seemingly discordant pharmacological results; while the NMDA receptor antagonists, MRZ2/579 (i.p.) and memantine (i.p.), inhibited the acquisition of morphine (i.v.) self-administration, NMDA receptor antagonist MK-801 (i.p.) did not affect morphine self-administration. The difference between memantine/MRZ2/579 and MK801 is potentially attributable to differences in affinity for the receptor. MK-801 is a high-affinity NMDA receptor antagonist with widely noted motor side effects in rat and mouse including the ICR mouse strain used in the present study (Fairbanks et al. 2000). Memantine and MRZ2/579 are considered to be low affinity NMDA receptor antagonists with K_i values in the low μM range (Parsons et al. 1999). Like memantine and MRZ2/579, agmatine demonstrates a low affinity for the NMDA receptor in terms of its ability to compete with [3H]-MK801 (Reynolds et al.

1990). Also, like memantine (Parsons et al. 1999), agmatine demonstrates a significantly improved side-effect profile relative to MK801 in pre-clinical mouse models (accelerating rotarod assay) (Fairbanks et al. 2000).

The role of nitric oxide in opioid self-administration has yet to be fully elucidated. Kivastik and colleagues (Kivastik et al. 1996) demonstrated the effects of N(G)-nitro-L-arginine (L-NOARG), a NOS inhibitor, on morphine-conditioned place preference and found that intraperitoneally delivered L-NOARG decreased the amount of time spent in the drug-paired side. Alternatively, Sarhaei and colleagues (Sarhaei et al. 2004) found that the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) (i.p.) increased morphine self-administration, the nitric oxide (NO) precursor L-arginine decreased morphine self-administration and supports morphine self-administration. These observations may result from NO-induced release of dopamine from striatal neurons (Kiss 2000).

It is significant that the most effective agmatine dose (10 nmol, i.c.v.) is within the range of agmatine doses that inhibit NMDA-evoked scratching and biting behavior when given spinally (Fairbanks and Wilcox 2000). It is also a dose that has been shown to inhibit NMDA-evoked thermal tail flick hyperalgesia (Fairbanks et al. 2000), a behavior that is dependent on NOS activation (Kitto et al. 1992). Agmatine's complete prevention of fentanyl-evoked self-administration is a typical observation of agmatine-mediated attenuation or reversal of glutamate-driven behavioral responses (Nguyen et al. 2003). Similar effects have now been reported in at least ten plasticity-related behaviors (Nguyen et al. 2003).

It is noteworthy that while we observed that pre-treatment of agmatine in the acquisition phase prevented the development of oral fentanyl self-administration, post-treatment with agmatine following establishment of fentanyl self-administration was not effective. This finding is consistent with other opioid related studies demonstrating that other NMDA receptor antagonists are effective in the development but not the maintenance phase of the opioid response specifically in opioid tolerance studies (Herman et al. 1995) and studies of opioid conditioned place preference (Papp et al. 2002; Ma et al. 2006) These outcomes also concur with evidence supporting the NMDA receptor in the induction, but not the maintenance phase of long-term potentiation in spinal cord (Benrath et al. 2005), hippocampus (Ohno et al. 2002), and cortex (Myers et al. 2000). That reinstatement of fentanyl self-administration was inhibited by i.c.v. agmatine is also an important finding that is consistent with other studies showing decreased CPP reinstatement following administration of other NMDAR antagonists (Popik et al. 2006). That extinction may influence the maintenance of long-term potentiation (LTP), it is likely that agmatine influences the induction of LTP. Therefore, the pattern of agmatine inhibition of oral fentanyl self-administration that we have observed is consistent with a general relationship of NMDA receptor antagonists in plasticity related events in a broad spectrum of CNS regions.

It has been previously noted that agmatine shares many of the classic characteristics of a modulator of neurotransmission (Reis and Regunathan 1998). Consequently, agmatine may act as an anti-glutamatergic modulator in vivo, a role largely unexplored in CNS. Further, we have recently shown Ca^{2+} -dependent

depolarization-evoked release of [³H]-agmatine from synaptosomes (Goracke-Postle et al. 2006; Goracke-Postle et al. 2007). Additionally, it has been observed that agmatine concurrently inhibits both glutamate release and seizures evoked by pentylentetrazole in the rat (Feng et al. 2005). Together with the pharmacological data (Nguyen et al. 2003) these observations support the proposal that endogenous agmatine may serve as a neuromodulator of the glutamatergic system (Reis and Regunathan 1998). Further studies are required to determine whether supraspinal endogenous agmatine prevents induction of opioid-induced self-administration in an NMDA receptor/NOS dependent manner.

Chapter IV

Immunoneutralization of Agmatine Sensitizes Mice to Mu-Opioid Receptor Tolerance

The development of opioid tolerance is a hallmark of addiction to drugs that are readily self-administered (Lee and Messing 2008; Goforth et al. 2010), including that of fentanyl (Kosten and George 2002) and is also thought to be mediated by the NMDA/NOS cascade. As an endogenous neuromodulator of this cascade, agmatine has the potential to mediate these events. This study focuses on the role of endogenous agmatine in acute opioid tolerance. We tested the following hypothesis: Sequestration of endogenous agmatine increases opioid tolerance.

The following data was published:

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(C.L.W. planned and conducted the experiments and wrote the paper. L.L.E., H.O.X.N., and K.F.K., conducted experiments.. G.L.W. assisted with method development, interpretation and editing. C.A.F. assisted with method development, interpretation and editing.)

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Systemically or centrally administered agmatine (decarboxylated arginine) prevents, moderates, or reverses opioid-induced tolerance and self-administration, inflammatory and neuropathic pain, and sequelae associated with ischemia and spinal cord injury in rodents. These behavioral models invoke the NMDA receptor/nitric oxide synthase cascade. Agmatine (AG) antagonizes the NMDA receptor and inhibits nitric oxide synthase *in vitro* and *in vivo*, which may explain its effect in models of neural plasticity. Agmatine has been detected biochemically and immunohistochemically in the central nervous system. Consequently, it's conceivable that agmatine operates in an anti-glutamatergic manner *in vivo*; the role of endogenous agmatine in the CNS remains minimally defined. The present studies used an immunoneutralization strategy to evaluate the effect of sequestration of endogenous agmatine in acute opioid analgesic tolerance in mice. First, intrathecal pre-treatment with an anti-AG IgG reversed an established pharmacological effect of intrathecal agmatine: antagonism of NMDA-evoked behavior. This result justified the use of anti-AG IgG to sequester endogenous agmatine *in vivo*. Second, intrathecal pre-treatment with the anti-AG IgG sensitized mice to induction of acute spinal tolerance with two mu-opioid receptor-selective agonists, DAMGO and endomorphin-2. A lower dose of either agonist that, under normal conditions, produces moderate or no tolerance was tolerance-inducing following intrathecal pre-treatment of anti-AG IgG. The effect of the anti-AG IgG lasted for at least 24 hours in both NMDA-evoked behavior and acute opioid tolerance. These results suggest that endogenous spinal agmatine may moderate glutamate-dependent neuroplasticity.

Introduction

Decarboxylated arginine (agmatine) has been identified in the mammalian central nervous system (CNS) both biochemically (Li et al. 1994; Raasch et al. 1995; Fairbanks and Wilcox 2000) and neuroanatomically (Fairbanks et al. 2000). Agmatine was discovered as a clonidine-displacing substance (Li et al. 1994) which bound, but did not activate or inhibit, alpha-2 adrenergic receptors (Pinthong et al. 1995). However, agmatine also acts as a NMDA receptor antagonist at polyamine site (K_i: 15 μM, Gibson et al, 2002) and the MK801 binding site (Reynolds et al. 1990). Agmatine also inhibits (Galea et al. 1996) or inactivates (Demady et al. 2001) nitric oxide synthase. Electrophysiological (Yang and Reis 1999) and pharmacological (Fairbanks and Wilcox 2000; Roberts et al. 2005) evidence supports agmatine antagonism/inhibition at both NMDA receptor and nitric oxide synthase, proteins known as essential components of glutamatergic neurotransmission. This dual activity raises the question as to whether agmatine functions endogenously as an anti-glutamatergic neuromodulator. This proposed role for agmatine is also suggested from reports describing agmatine-mediated inhibition of opioid tolerance (Kolesnikov et al. 1996; Fairbanks and Wilcox 1997), opioid self-administration (Morgan et al. 2002), inflammation- and neuropathy-induced hyperalgesia (Fairbanks et al. 2000), behavioral sequelae following spinal cord injury (Fairbanks et al. 2000; Gilad and Gilad 2000; Yu et al. 2000) and evoked seizure (Feng, 2005).

It has been suggested (Reis and Regunathan 1998) that agmatine meets several criteria characteristic of an endogenous neuromodulator, including synthesis of agmatine in the

brain (Reis and Regunathan 1998), localization to neurons and synaptic vesicles (Otake et al. 1998), transport into nerve terminals (Sastre et al. 1997), release by depolarization (Reis and Regunathan 1998), transport into astrocytes (Regunathan et al. 1995), and enzymatic degradation by CNS agmatinase (Sastre et al. 1996). An important criterion yet to be tested includes a demonstration that endogenous agmatine performs the same physiological function as does exogenously administered agmatine. This criterion has been tested for a wide variety of neurotransmitters and neuropeptides including β -endorphin (Guerrero-Munoz et al. 1979), endomorphin (Zadina et al. 1997), anandamide (Devane et al. 1992), substance P (Share and Rackham 1981), CGRP (Tan et al. 1994), met- and leu-enkephalin (Hardy and Haigler 1985) and small molecules such as norepinephrine (Hardy and Haigler 1985). In view of the ability of exogenously administered agmatine to prevent opioid analgesic tolerance (Kolesnikov et al. 1996; Fairbanks and Wilcox 1997), we hypothesized that endogenous agmatine participates in modulation of opioidergic processes. Previous studies of potential antinociceptive endogenous compounds have inferred the activity of these neuromodulators through the use of pharmacological antagonists *in vivo*. However, agmatine is itself a receptor antagonist and an enzyme inhibitor, rendering such a strategy inappropriate. We applied an immunoneutralization strategy using scavenger antisera to evaluate the physiological role of agmatine, a method previously utilized for other pharmacological antagonists (Vanderah et al. 1994; Tseng et al. 2000; Ohsawa et al. 2001). We hypothesized that sequestration of endogenous agmatine using a structure-specific anti-agmatine immunoglobulin (anti-AG IgG) antibody would invoke the induction of acute mu-

opioid receptor tolerance at doses that normally are not tolerance-inducing. Our objective was to assess the impact of spinal delivery of anti-AG IgG on acute homologous tolerance induced by both DAMGO and endomorphin-2 (endo-2) to determine if diminished availability of endogenous agmatine affected that process.

We show that exogenously applied agmatine prevents the induction of DAMGO- and Endo-2-induced acute spinal tolerance, as has been previously shown for morphine. The present study demonstrates that pre-treatment with anti-AG IgG (but not normal IgG) increases DAMGO- and Endo-2-induced acute spinal tolerance, supporting the proposal that endogenous agmatine exerts a modifying affect on mu-opioid receptor acute tolerance and providing a mirror image parallel to the studies using exogenous agmatine in the same paradigm.

Materials and Methods

Animals. Experimental subjects were Institute of Cancer Research (ICR) male mice (21-30 g, Harlan, Madison). Subjects were housed in groups of 8 in a temperature- and humidity-controlled environment and maintained on a 12 hr light/dark cycle with free access to food and water. These experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Chemicals. Agmatine sulfate, aminoguanidine, L-arginine, D-arginine, and NMDA were purchased from Sigma Chemical (St. Louis, MO). MK801 (dizolcipine) was a gift of Merck. Endo-2 (YPFF) was synthesized by the University of Minnesota's Microchemical Facility. DAMGO (D-Ala², NMe-Phe⁴, Gly-ol⁵]- enkephalin, mw 513.7) was purchased from Tocris Cookson (St. Louis, MO). All drugs were dissolved in 0.9% saline.

Intrathecal injection. Agmatine was administered intrathecally (i.t.) in conscious mice according to the method of Hylden and Wilcox (Hylden and Wilcox 1980). Briefly, the pelvic girdle (ileac crest) of the mouse is gripped firmly by the thumb and forefinger of the injector's non-dominant hand. The skin above the ileac crest is pulled tautly to create a horizontal plane where the needle is inserted. The needle is a 30 gauge, 1/2 inch sterile disposable needle connected to a 50 μ L Luer-hub Hamilton syringe. All injections were delivered in 5 μ L volume.

NMDA-induced nociceptive test. Nociceptive responsiveness was tested in the NMDA nociceptive test. A constant dose of NMDA (0.3 nmol) was injected intrathecally in order to produce approximately 40-60 behaviors (scratches and bites directed to the hindlimbs) in the first minute post-injection. Co-administration of agmatine dose-dependently inhibits those behaviors (Fairbanks and Wilcox 2000; Roberts et al. 2005). In these experiments, anti-AG IgG, pre-immune serum, normal guinea pig IgG, or saline were administered as pre-treatments prior to agmatine and NMDA injection.

L-Arginine-induced nociceptive behavioral responses. Biting and scratching responses were induced by a single intrathecal injection (0.3 nmol) of L-arginine. The animal's scratching and biting responses were counted for 90 seconds following injection.

Antinociception. In the opioid tolerance studies, thermal nociceptive responsiveness in opioid tolerance studies was assessed using the warm water (52.5°C) tail-immersion assay. Briefly, mice were gently wrapped in a soft cloth such that their tails were exposed, and three-quarters of the length of the tail was dipped into the warm water. Tail-flick latencies were obtained before drug application to establish a baseline response. To test

for analgesia, opioid agonists (DAMGO and endomorphin-2) were injected i.t. as 2.5 min pretreatments, respectively. A maximum cut-off of 12 sec was set to avoid tissue damage. The results were then expressed as a percent of the maximum possible effect (%MPE) according to the equation:

$$(1) \quad \% \text{ MPE} = (\text{Post-drug latency} - \text{Pre-drug latency}) / (\text{Cutoff} - \text{Pre-drug latency}) \times 100$$

Tolerance induction. Tolerance was induced in mice using the following protocol: Mice were injected intrathecally with a high dose of either DAMGO (0.6 pmol) or endo-2 (30 nmol) or saline respectively to induce tolerance (or 0.06 pmol or 10 nmol for doses that do not induce tolerance). Thirty minutes following the tolerance-inducing injection, the tail flick latencies of the mice had returned to baseline. At that point mice were injected with either DAMGO or endo-2 at varying doses to create an analgesic dose-response curve.

Dose-response analysis. The ED₅₀ values and 95% confidence intervals (95% CIs) of drugs were calculated using the graded dose-response curve method of Tallarida and Murray (Tallarida and Murray 1987). A minimum of three doses were used for each drug. When evaluating the extent of a potency shift between treatment groups, a potency ratio was calculated. These calculations were performed using the pharmacological statistics software FlashCalc version 4.3.2 (Dr. Michael Ossipov, University of Arizona, Tucson, AZ).

Anti-Agmatine antisera generation. Agmatine sulfate was coupled to bovine thyroglobulin (BTG) with glutaraldehyde. The conjugate was dialyzed to remove excess glutaraldehyde. The conjugate was frozen in aliquots of 1 mg/mL concentration and

stored at -20°C for use in immunizations. Pre-immune serum was collected from four female guinea pigs (Duncan Hartley) prior to the first intradermal immunization with AG-BTG conjugate which was mixed as an emulsion in a 1:1 ratio with Complete Freund's adjuvant. Subsequent immunizations were administered every two weeks using incomplete Freund's adjuvant in a 1:1 ratio with the AG-BTG conjugate. After six weeks microliter quantities of sera were collected biweekly (alternating with the immunoboosts) and screened for immunoreactivity. After eleven weeks, the guinea pigs were anesthetized and exsanguinated by cardiac puncture. Blood was centrifuged, serum collected, aliquoted (1 mL) and stored at -80°C . Initial screening of the antisera by immunohistochemistry (IHC) from three of the four guinea pigs (GP1, 3, 4) showed similar patterns of immunofluorescence with some variation in background and intensity. Sera from the second guinea pig did not appear immunoreactive. Samples of the stored serum were subsequently thawed and subjected to protein-A column purification to reduce the samples to the IgG fraction, aliquoted and stored at -20°C . There is added value in using protein A-purified IgG (rather than serum or plasma) for immunoneutralization studies, particularly in neuroscience, as both glutamate and glycine (both ligands of the NMDA receptor) are present in serum and plasma at concentrations that could act upon NMDA receptors in rodents when injected intrathecally. Additionally, antisera for all four guinea pigs were screened in the bioassay described in Figure 1 representing agmatine inhibition of NMDA-elicited behavioral responses; the IgG from GP1, 3 and 4 reversed agmatine-induced inhibition of NMDA responses whereas IgG from GP2 did not (consistent with the evaluation by IHC). In the present study, the data

presented were obtained from the third guinea pig (GP3), which appeared to have the best response in both the immunoassay and behavioral bioassay. A sample of the protein-A purified IgG from GP3 was further purified by affinity purification using the ImmunoPure Immobilized Protein A column (Pierce, Rockford, IL) according to the manufacturer's protocol. Samples of the eluents from both the protein A purification and the affinity purifications were confirmed to contain IgG through ELISA identification on Protein A-preloaded ELISA plates purchased from Pierce.

Immunohistochemistry. Male rats (Sprague-Dawley, Harlan, WI; 120-150g) were deeply anesthetized (75 mg/kg ketamine, 5 mg/kg xylazine and 1 mg/kg acepromazine, i.m.) and fixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate-buffered saline (pH 6.9) by vascular perfusion. Spinal cords were removed and placed in 10% sucrose in phosphate-buffered saline overnight at 4°C. Spinal segments were frozen and thaw-mounted cryostat sections (14 µm) prepared for indirect immunofluorescence histochemistry. The sections were pre-incubated for one hour at room temperature in diluent containing 1% normal donkey serum, 0.3% Triton X-100, 0.01% sodium azide and 1% bovine serum albumin. Sections were then incubated overnight in a humid chamber with primary antisera and rinsed several times with phosphate-buffered saline. Sections were then incubated with secondary antisera for one hour at room temperature, rinsed and cover-slipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Other primary antisera used were: rabbit-derived anti-AG (a gift Dr. Donald Reis, dilution 1:50), rabbit-derived anti-GFAP (1:50 ICN Biomedicals (now MP Biomedicals, Irvine California)), mouse-derived anti-NeuN (1:500, Chemicon, Temecula,

CA). Preparations were visualized with RedX rhodamine-conjugated secondary antisera (1:200; Jackson ImmunoResearch, West Grove, PA) and examined with a Bio-Rad MRC-1000 Confocal Imaging System (Bio-Rad Microscience Division, Cambridge, MA). Micrographs were assembled using Photoshop 7.0 (Adobe).

Experimental standards. All experiments were repeated at least once by blinded investigators and showed consistent results.

Results

Anti-Agmatine immunoreactivity. To develop an antiserum to agmatine, guinea pigs were immunized with a conjugate of agmatine and bovine thyroglobulin (BTG) and polyvinylidene fluoride transfer membrane was spotted with agmatine sulfate-BTG conjugate. Dot blots were probed with the protein A-purified anti-AG IgG in the presence or absence of varying concentrations (10, 1.0, 0.1 mM) of agmatine and its precursor, L-arginine. The antibody binding to the polyvinylidene fluoride membrane was diminished by agmatine, but not L-arginine, in a concentration-dependent manner, indicating the specificity of agmatine for the IgG (data not shown).

We detected agmatine immunoreactivity (AG-IR) in rat spinal cord with guinea pig-derived anti-AG IgG which co-labeled spinal structures with the rabbit-derived anti-AG antiserum used in previous reports (Otake et al. 1998; Fairbanks et al. 2000). The pattern of AG-IR included labeling of structures surrounding the perikarya of anti-NeuN-identified spinal and glial fibers, consistent with previous reports that agmatine may be present in glia (Regunathan et al. 1995). AG-IR was not observed in tissue treated with pre-immune serum (data not shown) and the AG-IR observed in spinal structures was

concentration-dependently diminished following pre-incubation of antiserum with free agmatine sulfate, but not L-arginine (data not shown). Immunoreactivity to other antisera (neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP)) was not changed by pre-incubation with agmatine, a result that ensures that the observed agmatine absorption control was not due to a non-specific impact of agmatine sulfate on other IgG immunoreactivity (data not shown).

***In vivo* specificity of anti-AG IgG for agmatine.** We conducted a series of studies to examine the *in vivo* effects of the anti-AG IgG upon delivery of either NMDA alone or NMDA + AG in an established model of NMDA-evoked biting and scratching behaviors (Fairbanks et al., 2000). Consistent with previous reports (Fairbanks et al. 2000; Roberts et al. 2005) agmatine (60 nmol, i.t.) significantly reduced NMDA-evoked biting and scratching behavior (Fig 1A-C, bars 1 and 2). In contrast, pre-treatment with the anti-AG IgG showed a significant reversal of the agmatine-mediated inhibition of NMDA-evoked behavior, supporting the concept of IgG-mediated agmatine sequestration (Table 1). Heating the anti-AG IgG to 100 °C for five minutes prior to intrathecal administration abolished this effect (data not shown), indicating this is dependent upon the intact anti-AG IgG protein structure. In the absence of agmatine, NMDA responses did not differ in subjects pre-treated with either pre-immune serum or anti-AG IgG (data not shown). The anti-AG IgG also showed a dose-dependence with the most effective dose being 150 ng (Figure 1A, bars 3-5). To determine what part of the agmatine molecule may bind to the IgG, the same experiment was conducted using aminoguanidine (Fig. 1B), the chemical structure of which is a guanidino group, a constituent of the agmatine molecule.

Aminoguanidine (10 nmol, i.t.) significantly reduced NMDA-evoked behavior. As in the agmatine experiment, pre-treatment with the anti-AG IgG showed a dose-dependent reversal of aminoguanidine-mediated inhibition of NMDA behavior suggesting that the guanidino group may be important in the binding of agmatine to both the NMDA receptor and the IgG (Fig. 1B). To control for a potential non-specific IgG effect on the NMDA receptor or downstream signaling that governs that behavioral response, the same experiment was conducted with MK801 (Fig. 1C), an NMDA receptor antagonist with a chemical structure unrelated to that of agmatine. MK801 (1 nmol, i.t.) significantly reduced NMDA-evoked biting and scratching behavior. Pre-treatment with the anti-AG IgG showed no effect upon MK801-mediated inhibition of NMDA behavior, arguing against a non-specific effect of the IgG on the NMDA receptor or downstream mediators. Finally, we evaluated the possibility that the IgG could bind to L-arginine, which also contains a guanidino group (Fig. 1D). Intrathecal injection of L-arginine (600 nmol) elicits a scratching and biting behavior similar to NMDA. Pre-treatment with the anti-AG IgG showed no effect on L-arginine-evoked behavior up to 300 ng. Taken collectively, these *in vivo* bioassays (Fig 1A-D) indicate the *in vivo* specificity of the anti-AG IgG for the target molecule, agmatine.

We next studied the duration of action for the anti-AG IgG. Either anti-AG IgG or saline was given as 1 min, 24 hour, or 48 hour pre-treatments prior to co-administration of the NMDA and agmatine (Fig. 2). As expected, agmatine effectively inhibited the responses in mice that had been pre-treated with saline at 1 min, 24 and 48 hours (light grey bars). However, agmatine did not inhibit NMDA-evoked responses in mice pre-treated with

anti-AG IgG at 1 minute or at 24 hours (1st and 2nd dark bars, Fig. 2). This reversal was no longer observed at the 48-hour pre-treatment time point. Therefore, the anti-AG IgG pre-treatment appeared to reverse the effect of agmatine for at least 24 hours.

Agmatinerbic modulation of DAMGO- and Endo-2-induced acute tolerance. Figure 1 shows that anti-AG IgG reversed exogenous agmatine-induced (but not MK801-induced) inhibition of NMDA-evoked behavior in mice, suggesting specificity of the anti-AG IgG for the target molecule. This provided the rationale to test the anti-AG IgG in a model of opioid receptor agonist-induced plasticity. It had been previously shown that acute analgesic tolerance develops to supramaximal doses of intrathecally-administered morphine in an NMDA-receptor/NOS-dependent manner (Fairbanks and Wilcox 1997). In that study it was also shown that intrathecally administered agmatine prevented acute spinal morphine tolerance (Fairbanks and Wilcox 1997). We hypothesized that sequestration of endogenous agmatine by intrathecal administration of anti-AG IgG should reduce inhibitory tone upon development of acute opioid tolerance, sensitizing subjects to induction of acute tolerance by lower doses of opioid that normally induce moderate amounts of tolerance.

We tested agmatine for blockade of acutely induced tolerance to intrathecal DAMGO (Fig 3A) and endo-2 (Fig 3B). To characterize acute spinal DAMGO and endo-2 tolerance, we determined the antinociceptive dose-response curves in saline-, DAMGO (0.6 pmol, i.t.)- and endo-2 (30 nmol, i.t.)-pretreated mice. While the mu-opioid agonists produced dose-dependent antinociception in saline-pretreated mice, pre-treatment with either DAMGO (Fig. 3A) or endo-2 (Fig. 3B) significantly reduced the efficacy of the

respective agonists at all doses tested, confirming induction of acute tolerance. When agmatine (4 nmol, i.t.) was administered as a co-pretreatment with the same tolerance-inducing doses of the agonists, acute tolerance was prevented (Fig. 3A, B). The probe agonist dose-response curves in DAMGO-AG or endo-2-AG pre-treated subjects resulted in an ED₅₀ value comparable to that of the saline pre-treatment group and significantly different from the agonist pre-treatment group (Fig. 3A and B, Table 1 and 2). This result demonstrates that agmatine robustly attenuates acutely induced tolerance to the spinally administered mu-opioid agonists DAMGO or endo-2.

The doses of DAMGO and endo-2 used to induce analgesic tolerance was 0.6 pmol and 30 nmol, respectively. Having observed a pharmacological effect for exogenous agmatine, we next evaluated the impact of pre-treatment with the anti-AG IgG with administration of lower doses of DAMGO (0.06 pmol) and endo-2 (10 nmol) that did not evoke analgesic tolerance (Fig. 3C and D). To characterize the impact of anti-AG IgG pre-treatment on acute spinal opioid-induced tolerance, we determined the antinociceptive dose-response curves in mice pre-treated with the mu-opioid agonists or co-pretreated with the mu-opioid agonists and either normal guinea pig IgG or anti-AG IgG. When normal guinea pig IgG was administered prior to the DAMGO (0.06 pmol) or endo-2 (10 nmol) low-dose pre-treatment, the probe agonist dose-response curves are comparable to that of the saline-treated mice, indicating that normal IgG has no effect on the analgesic dose-response curves of DAMGO or endo-2. However, when anti-AG IgG is administered prior to either DAMGO or endo-2 low dose pre-treatment, the subsequent DAMGO and endo-2 probe analgesic potency is lower than the saline or normal IgG pre-

treatment groups (Table 2 and 3). These decreases in potency are significant and suggest that anti-AG IgG sensitized the mice to mu-opioid agonist-evoked spinal tolerance. Therefore, the anti-AG IgG sensitization of mice to acutely induced DAMGO tolerance is not a non-specific effect of pre-treatment with IgG.

The data presented in Figure 3B and D show that pre-treatment with the anti-AG IgG causes the lower dose of agonist to induce tolerance; at this dose, tolerance is not observed with control pre-treatments of either saline or normal guinea pig IgG. This result supports the concept that reduction of endogenous agmatine by anti-AG IgG sensitizes subjects to induction of spinal opioid tolerance peptides.

As a control for potential anti-AG IgG effects on acute endo-2 antinociception, the anti-AG IgG was administered in one treatment group *after* the endo-2 pre-treatment. In other words, it was given as a co-treatment with the endo-2 probe dose (10 nmol). The resulting antinociceptive response (%MPE: 71 ± 9.1 , $n = 7$) was comparable to responses observed from the administration of probe doses of endo-2 (10 nmol) to the groups pre-treated with saline (%MPE 88 ± 5.5 , $n = 8$) or 10 nmol endo-2 (%MPE: 74 ± 8.8 , $n = 8$), but different from the group pre-treated with anti-Ag IgG + 10 nmol endo-2 (%MPE: 27 ± 9.8 , $n=8$). These results demonstrate that the sensitizing effect of the anti-Ag IgG, is on the endo-2 pre-treatment-induced tolerance component of the experiment rather than a putative antagonizing effect at the time of the endo-2 probe.

It was of interest to determine the duration of the effect of the anti-AG IgG administration in the opioid tolerance assay. In order to assess that, anti-AG IgG was delivered as a co-treatment, or as 1 minute, 24 hour, or 48 hour pre-treatments before

induction of endo-2 tolerance. The first bar of Fig. 4 shows a typical 70% MPE analgesic response for a 10 nmol dose of endo-2 following a 30 minute Endo-2 pre-treatment (also 10 nmol). This result shows that there was no induction of tolerance by a pre-treatment of 10 nmoles. The second bar shows that pre-treatment with normal guinea pig serum (150 ng) and the same dose of endo-2 (10 nmol) did not alter the analgesic response of endo-2 given 30 minutes later. However, consistent with the data profiled in Fig. 3B, a pre-treatment of anti-AG IgG (150 ng) given with the 10 nmol dose of endo-2 results in a significantly diminished analgesic response to the probe dose of endo-2 (third bar), presumably sensitizing the subjects to opioid-induced tolerance. Furthermore, the 4th, 5th, and 6th bars respectively show that, when the anti-AG IgG pre-treatment is administered to the mice 15 min, 24 and 48 hrs prior to administration of the endo-2 pre-treatment (contrasted with administering the anti-AG IgG at the same time), the anti-AG IgG still invokes sensitization to the development of acute opioid tolerance represented by an apparent analgesic tolerance to the low dose of endo-2 (10 nmol). Therefore, the anti-AG pre-treatment appeared to sensitize the mice to opioid tolerance for up to 48 hours.

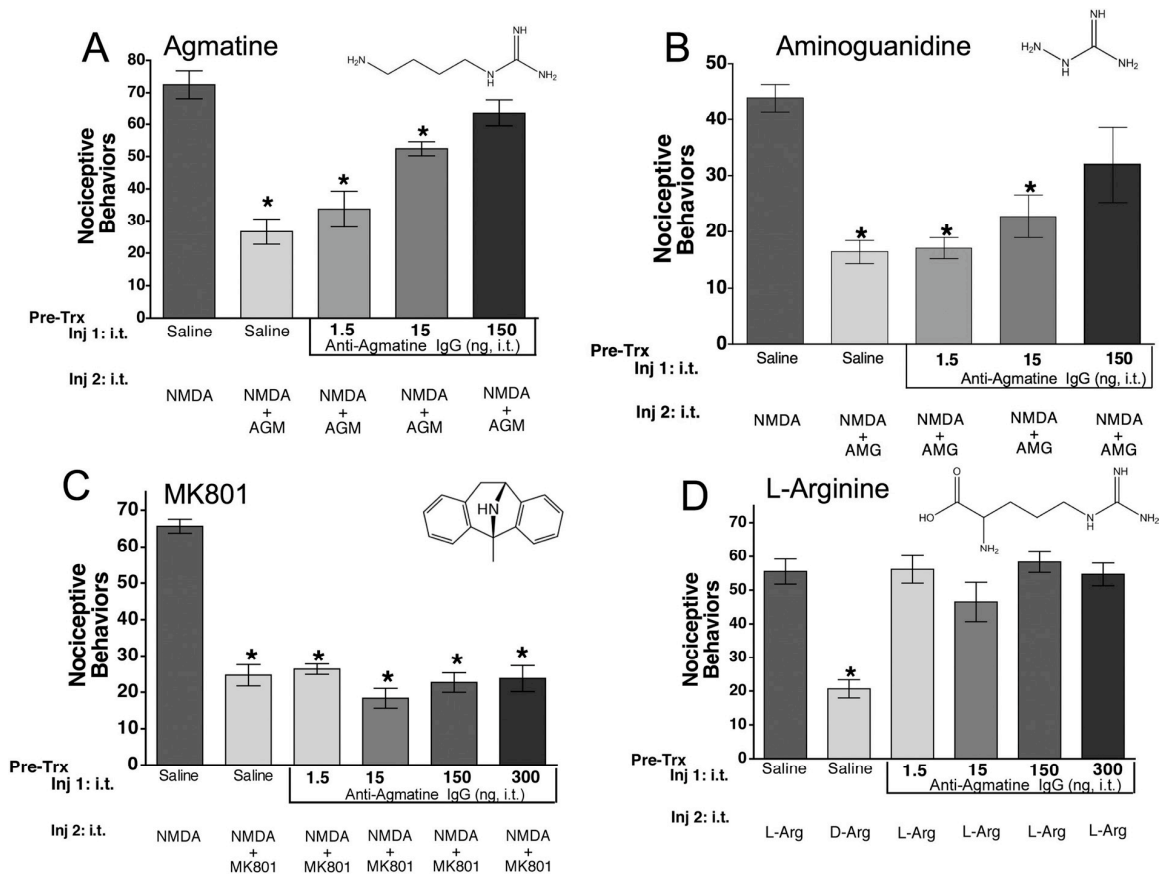


Fig. 1. *In vivo* behavioral specificity of anti-AG IgG for agmatine. A, B, C, and D illustrate the impact of anti-AG IgG on modulation of NMDA-evoked behavior by agmatine-, MK801-, and aminoguanidine as well as on similar behaviors evoked by L-arginine in mouse spinal cord. In A, B, and C, NMDA (0.3 nmol, i.t.) produced scratching and biting behaviors (first bars of Panels A, B, and C). Agmatine (60 nmol, i.t.), MK801 (1 nmol, i.t.), and aminoguanidine (1 nmol, i.t.) all equi-effectively block NMDA-evoked behavior (second bars of Panels A, B, and C). Five minute pre-treatment with anti-AG IgG dose-dependently reverses agmatine (A) and aminoguanidine (B). but

not MK801 (C) inhibition of NMDA-evoked behavior. Panel D shows that L-arginine (but not D-arginine) produces scratching and biting behaviors similar to NMDA (first and second bars of Panel D). Five minute pre-treatment with anti-AG IgG did not impact L-arginine-induced scratching and biting behaviors even at twice the dose effective for reversal of aminoguanidine and agmatine effects. *indicates significance ($p < 0.05$) as evaluated by ANOVA followed by Dunnett's posthoc test for multiple comparisons to a control: A) $F_{(4,35)} = 27$; B) $F_{(4,35)} = 12$; C) $F_{(5,42)} = 76$; D) $F_{(5,42)} = 18$.

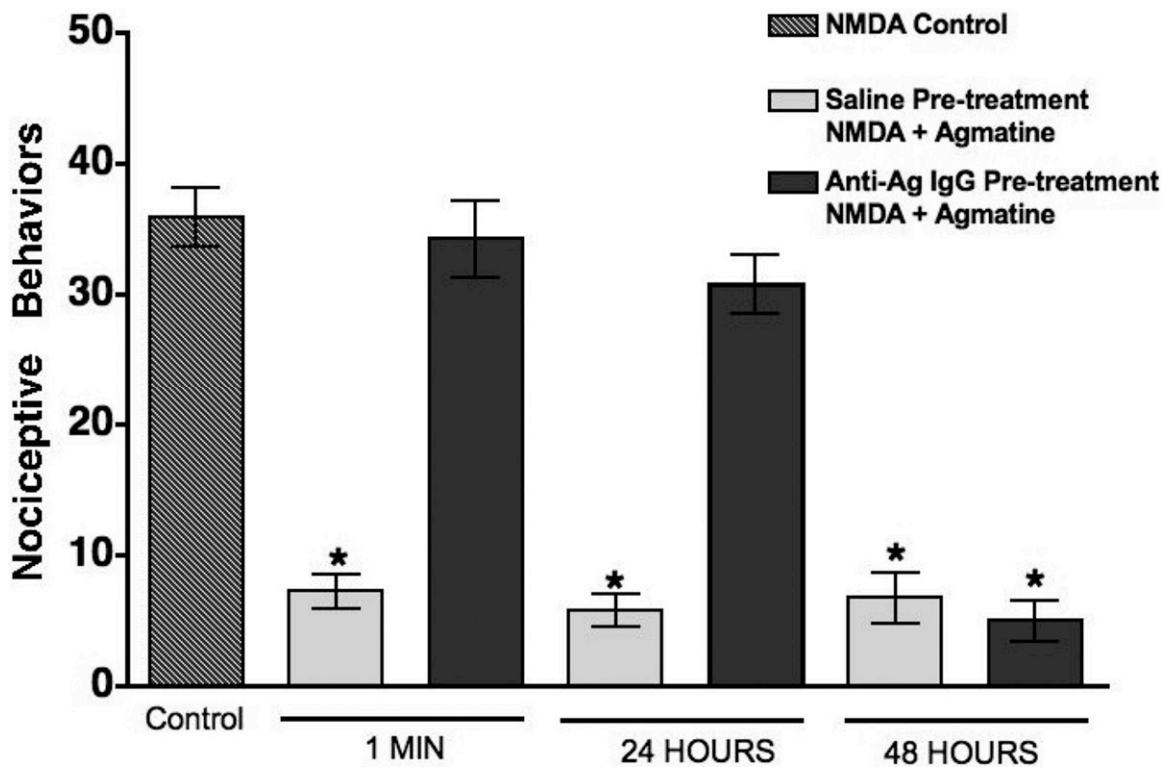


Fig. 2. Duration of Anti-AG IgG effect on the Agmatine Inhibition of NMDA-evoked responses. Anti-AG IgG effectively reverses agmatine inhibition of NMDA-evoked behavior when given as a 1 minute and 24 hour (but not 48 hour) pre-treatment. Striped bar represents NMDA (0.3 nmol) injection to establish the baseline number of behaviors. Black bars represent a saline injection given at various pre-treatment times, followed by an NMDA + Agmatine (60 nmol, i.t.) co-injection. Grey bars represent anti-AG IgG injection (150 ng) given at various pre-treatment times followed by an NMDA + Agmatine (60 nmol, i.t.) co-injection. *indicates significance ($p < 0.05$) as evaluated by ANOVA followed by Dunnett's posthoc test for multiple comparisons to a control: $F_{(6,49)} = 86$.

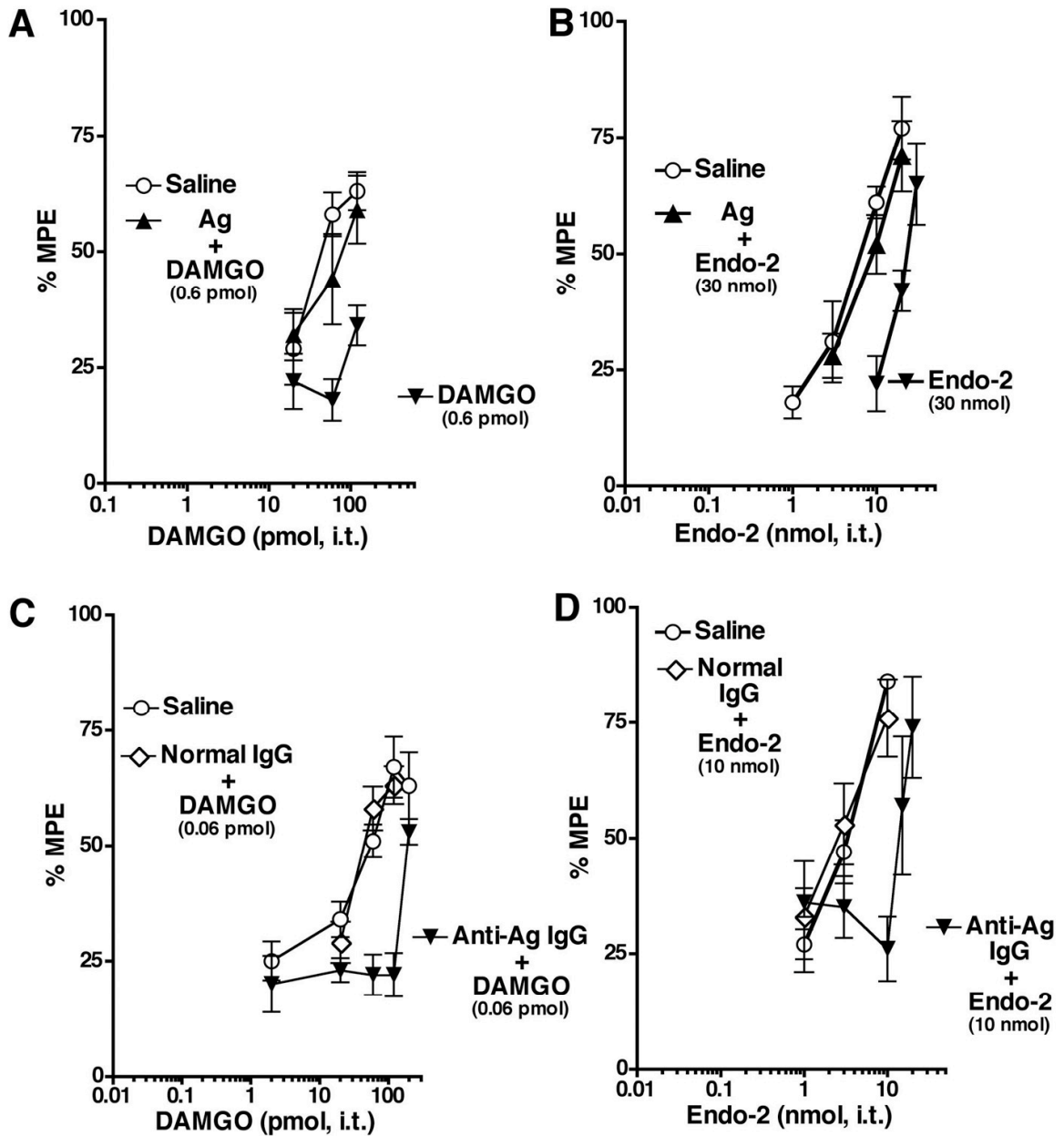


Fig. 3. Agmatinergetic Effects on Acute DAMGO and Endo-2 Analgesic Tolerance.

(A) Pretreatment with DAMGO (0.6 pmol, i.t., inverted triangles) decreased DAMGO potency and efficacy compared to saline-treated controls (open circles), indicating

induction of acute opioid analgesic tolerance. Co-administration (upright triangles) of agmatine (4 nmol, i.t.) with this same tolerance-inducing dose of DAMGO (0.6 pmol) prevented the induction of DAMGO acute tolerance. **(B)** Pre-treatment with endomorphin-2 (30 nmol, i.t., inverted triangles) decreased endo-2 potency compared to saline-treated controls (open circles), indicating induction of acute opioid analgesic tolerance. Co-administration of agmatine (4 nmol, i.t.) with this same tolerance-inducing dose of endo-2 (30 nmol) prevented induction of acute endo-2 tolerance (upright triangles). **(C)** The response to DAMGO in mice co-pretreated with normal guinea pig IgG + low dose DAMGO (0.06 pmol, diamonds) was comparable to that of mice pretreated with the saline (circles) indicating lack of induction of acute tolerance. In contrast, the DAMGO analgesic response in mice that received a co-pre-treatment with anti-AG IgG + DAMGO (0.06 pmol, inverted triangles) was of significantly lower potency. **(D)** In mice pre-treated with a lower dose of endo-2 (10 nmol, i.t., upright triangles) the endo-2 analgesic potency was comparable to saline-treated controls (circles), indicating lack of induction of acute tolerance. In contrast, the endo-2 response in mice that received a co-pretreatment with anti-AG IgG + endo-2 (10 nmol, inverted triangles) was of significantly lower potency.

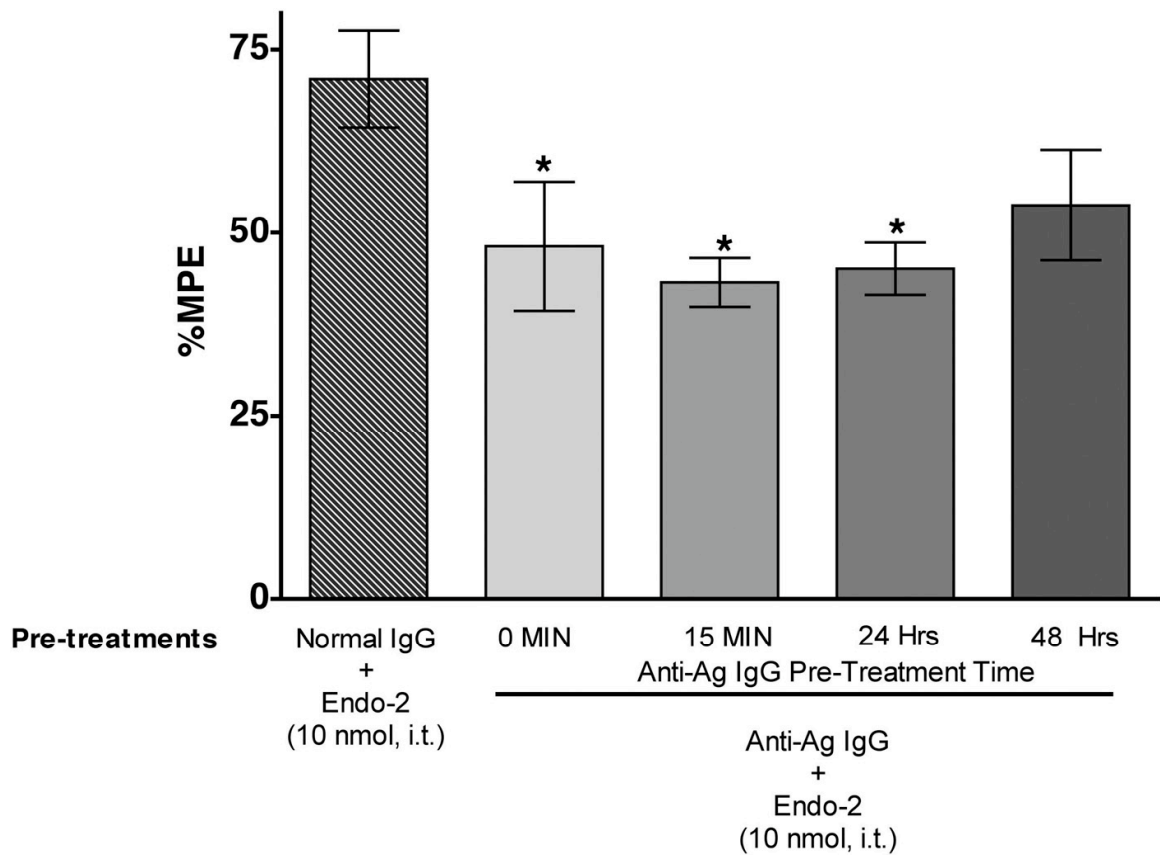


Fig. 4. Duration of Agmatinergetic Effects on Acute Endo-2 Analgesic Tolerance. Anti-AG IgG effectively sensitizes mice to acute endomorphin-2 analgesic tolerance when given as a 1 minute, 24 hour, or 48 hour pre-treatment. When normal guinea pig serum is given with the pre-treatment of endo-2 (10 nmol), there is no impact on the level of analgesia (first bar). However, when anti-AG IgG is given as a co-treatment (2nd bar) or as a pre-treatment (3rd, 4th bars) to endo-2, antinociception is significantly diminished relative to the normal guinea pig IgG-pretreated control. *indicates significant difference from the endo-2 + Normal GP Serum pre-treatment group (P <0.05), both measures were

evaluated by ANOVA (Dunnett's post-hoc test for multiple comparisons to a control).

$$F_{(4,45)} = 3.4.$$

Table 1. ED₅₀ values for Fig. 3A and C pre-treatment group

Pre-treatment Group	Probe DAMGO ED₅₀ (95% CI) (pmoles, i.t.)	Relative Potency (95% CI) (compared to saline)	Pre-treatment Group	Probe DAMGO ED₅₀ (95% CI) (pmols, i.t.)	Relative Potency (95% CI) (compared to saline pre-treatment)
Saline	51 (34-75)	1	Saline	52 (39-68)	1
DAMGO (0.6 pmol)	Not calculable	Not calculable	Normal IgG + DAMGO (0.6 pmol)	50 (32-77)	0.9 (0.58-1.6)*
DAMGO (0.6 pmol) + Agmatine (4 nmol)	75 (39-140)	1.5 (0.69-3.1)	Anti-AG IgG + DAMGO (0.6 pmol)	4600 (1800-12,000)	89 (34-240)*

*indicates significant difference relative to the saline pre-treatment group

Table 2. ED₅₀ values for Fig. 3B and D pre-treatment groups

Pre-treatment Group	Probe ENDO-2 ED₅₀ (95% CI) (nmol, i.t.)	Relative Potency (95% CI) (compared to saline)	Pretreatment Group	Probe ENDO-2 ED₅₀ (95% CI) (nmoles, i.t.)	Relative Potency (compared to saline)
Saline	5.8 (4.3-8)	1	Saline	3.0 (2.3-4.0)	1
ENDO-2 (30 nmol)	22 (18-27)	3.7 (2.5-7.9)*	Normal IgG + ENDO-2 (10 nmol)	2.8 (1.7-4.8)	0.9 (0.51-1.7)
ENDO-2 (30 nmol) + Agmatine (4 nmol)	8.2 (5.9-12)	1.4 (0.9-2.2)	Anti-AG IgG + ENDO-2 (10 nmol)	14 (12-16)	4.6 (3.3-6.4)*

*indicates significant difference relative to the saline pre-treatment group.

Discussion

The current study examined the effect of endogenous agmatine in a model of acute opioid tolerance. It has been shown by a number of research groups that exogenously administered agmatine prevents opioid-induced analgesic tolerance (for review, Nguyen, 2003). Such evidence suggests that endogenous agmatine could moderate the development of opioid-induced analgesic tolerance. Using the anti-agmatine IgG sequestration approach, it was observed that manipulation of free levels of agmatine in the spinal cord allowed lower doses of intrathecal opioid to evoke acute spinal analgesic tolerance. This result provides proof of concept for the endogenous role of agmatine as a modulator of spinal neural plasticity.

Other research groups have shown that antisera to endogenous compounds can be used to interfere with the actions of endogenous compounds in *in vivo* models of opioid tolerance and analgesia, including neuropeptide FF (Lake et al. 1991), Leu and Met-enkephalin (Vanderah et al. 1994; Tseng et al. 2000; Ohsawa et al. 2001), β -endorphin (Tseng et al. 2000; Ohsawa et al. 2001), and dynorphin (Ossipov et al. 1996; Tseng et al. 2000; Ohsawa et al. 2001).

The present study demonstrated that intrathecal pre-treatment with protein-A-purified agmatine IgG (e.g. antiserum purified to the IgG fraction) dose-dependently and specifically interferes with agmatine-induced inhibition of NMDA-evoked behavior. The anti-AG IgG dose-dependently reversed the ability of aminoguanidine to inhibit NMDA-evoked behavior, which is significant because aminoguanidine is in effect a guanidine group and a chemical constituent of the agmatine molecule; and as such, suggests an

epitope for the antibody. The guanidino moiety is also represented in the structure of the agmatine precursor, L-arginine. Consequently, the IgG the protein was evaluated for cross-reactivity to L-Arginine in this bioassay. However, pre-treatment with the anti-AG IgG did not impact L-arginine-evoked behaviors even at twice the effective dose. Therefore, it seems unlikely that the anti-AG IgG binds to L-arginine. Further, previously generated and studied anti-AG anti-sera raised in rabbit did not show L-ARG cross-reactivity in ELISA binding assays (Otake et al. 1998). Having documented proof-of-concept that agmatine can be selectively immunoneutralized *in vivo*, we next evaluated the effects of intrathecally injected antiserum in a model of acute spinal opioid tolerance using the opioid peptides DAMGO and Endo-2 (YPFF).

Anti-Agmatine IgG Effect on Spinal Mu-Opioid Receptor Tolerance. We showed that homologous tolerance induced by each of the two agents resulted in an approximately 10-fold decrease in analgesic potency of each agent compared to saline-pretreated controls. Co-administration of agmatine with the tolerance-inducing opioid prevented induction of opioid tolerance, consistent with previous reports (for review please see (Nguyen et al. 2003). Conversely, co-treatment with anti-AG IgG (but not normal IgG) permitted lower doses of DAMGO or endo-2 to become tolerance-inducing. This effect was not observed in animals that received a pretreatment of IgG from non-immunized guinea pigs (normal IgG). We interpret these results to suggest that sequestration of endogenous agmatine by agmatine-selective IgG increases susceptibility of mice to tolerance induced by these opioids. These data suggest that, under normal

conditions, endogenous agmatinerbic tone may control or dampen the development of mu opioid receptor tolerance.

A number of inhibitory neurotransmission systems (enkephalins, GABA, norepinephrine, endocannabinoids) are known to exert control over excitatory neurotransmission in CNS. Each of these agonists has a G_i -coupled 7 transmembrane receptor and, as such, have been readily characterized through neuropharmacological studies using selective antagonists such as naloxone, phaclofen, yohimbine, and SR 141716A. Prior reports that exogenous agmatine prevented glutamate-dependent behavioral events formed the rationale for the proposal that endogenous agmatine could exert some anti-glutamaterbic control. The present manuscript extends that result to include a parallel observation: that reduction of freely available agmatine could sensitize subjects to glutamaterbic events. As a putative inhibitor of glutamate neurotransmission, agmatine would also differ from the previous two putative endogenous NMDA receptor antagonists in that it may exert dual activity upon the NMDA receptor and/or its downstream signal transduction mediator, nitric oxide synthase. Further, unlike other glutamate mediators, agmatine is reported to have an uptake mechanism into purified nerve terminals (Sastre et al. 1997) and is also released from these structures by either K^+ or evoked depolarization (Reis and Regunathan 1998) or capsaicin exposure (Goracke-Postle 2007b). Consequently, the agmatinerbic influence of glutamaterbic neuromodulation may be of neuronal origin. However, because agmatine has also been shown to be synthesized and stored in astrocytes (Regunathan et al. 1995), the site(s) of cellular synthesis, uptake, and release of agmatine remains under investigation. Identification of such agmatinerbic

neurodynamics may be key to defining its role in chronic neuroplastic processes. The scope of the present study is limited to the action of the anti-agmatine IgG in an acute model of spinal opioid tolerance. We, and others, have reported that exogenous agmatine inhibits plasticity behavior accompanying opioid tolerance and addiction, chronic pain, and spinal cord injury (Nguyen et al, 2003). It is possible that endogenous agmatine plays a similar role in control of those processes. Evidence supports the role of glia in plasticity; the role of glia as a contributor to plasticity is increasingly appreciated, and the source of endogenous agmatine regulation may involve glia as well as neurons. The complexity of the system permits the participation of agmatine as an intensity control affecting plasticity events in the CNS.

Increasing evidence suggests that decarboxylated arginine, agmatine, operates as a novel neurotransmitter and/or neuromodulator in mammals (Reis and Regunathan 1998). Asserting that claim requires, in part, testing the hypothesis that, when administered exogenously, agmatine produces pharmacological effects comparable to the proposed physiological effects of the putative endogenous molecule. Since its discovery in the CNS (Li et al. 1994), there have been numerous studies evaluating a variety of physiological effects produced by exogenous administration of agmatine. To our knowledge, the present is the first to conduct a direct side-by-side comparison with exogenous administration of agmatine and a tool intended to neutralize endogenous agmatine. Further studies are needed that modulate endogenous agmatine levels in *in vivo* models in order to further define agmatinergetic CNS function.

Chapter V

Chronic sequestration of endogenous agmatine in fentanyl self-administration

The objective of this study is to determine the role of endogenous agmatine in plasticity-dependent behavioral phenomena using chronic sequestration of endogenous agmatine with an anti-agmatine IgG antibody. Our goal was to assess changes in fentanyl self-administration with chronic sequestration of agmatine. We tested the following hypothesis: The chronic absence of endogenous agmatine induces compensatory mechanisms through upregulation of arginine decarboxylase.

The following people contributed to this work:

Wade, Carrie L., Kitto, Kelley F., Schuster, Daniel J., Wilcox, George L., Fairbanks, Carolyn A.

(C.L.W. planned and conducted experiments. conducted the experiments, wrote paper. D.S. conducted experiments, K.F.K. conducted experiments. G.L.W. assisted with method development, interpretation and editing. C.A.F. assisted with method development, interpretation and editing.)

Opioid tolerance and self-administration has previously been shown to be modulated by NMDA receptor/NOS cascade. We have shown that morphine tolerance and fentanyl self-administration is modulated by a low-affinity NMDA receptor antagonist, agmatine (Wade et al. 2008; Wade et al. 2009). We developed a specific anti-agmatine antibody that when administered centrally (i.t. or i.c.v.) may immunoneutralize the level of endogenous agmatine present in the CNS. Using the anti-agmatine antibody we show that the anti-agmatine IgG increases susceptibility to acute opioid tolerance and decreases chronic opioid tolerance and self-administration. These seemingly paradoxical results may be explained by compensatory changes that occur in the agmatinergetic system following chronic opioid exposure. Mice administered the anti-agmatine antibody intracerebroventricularly (i.c.v.) show a decreased induction of morphine-evoked analgesic tolerance and a decreased rate of fentanyl self-administration. These results appear to be opioid specific; when given the anti-agmatine antibody i.c.v. under the conditions of food maintained responding, a non-opioid reinforcing paradigm, there was no change in response rates. To address the possibility of potential agmatinergetic compensatory changes, we examined the expression of arginine decarboxylase and agmatinase, the synthetic and degradative enzymes responsible for the synthesis and metabolism of agmatine following chronic delivery of anti-agmatine IgG given centrally.

Introduction

Agmatine, the cation formed by decarboxylated arginine, has been shown to prevent fentanyl self-administration and reduce the induction of opioid tolerance. Both of these opioid effects have been shown to be modulated by the glutamatergic system. Previously we have demonstrated a role for endogenous agmatine by acute sequestration of agmatine using an anti-agmatine antibody (Wade et al. 2009). Acutely, when sequestered, the absence of agmatine increases opioid induced tolerance by increasing tolerance to low doses of both morphine and endomorphin-2 (endo-2). Although we have elucidated a function for endogenous agmatine when actually absent, more useful information may be provided when agmatine is sequestered chronically. In this study, we gave the anti-agmatine IgG for a period of 16 days intracerebroventricularly (i.c.v.) (every other day) and tested mice in the fentanyl self-administration assay. We show that chronic administration of anti-agmatine IgG decreased induction of morphine-evoked analgesic tolerance and a decreased rate of fentanyl self-administration. These results appear to be opioid specific; when given the anti-agmatine antibody i.c.v. under the conditions of food-maintained responding, a non-opioid reinforcing paradigm, there was no change in response rates. These seemingly paradoxical results may be explained by compensatory changes that occur in the agmatinergetic system following chronic opioid exposure. To address the possibility of agmatinergetic compensatory changes, we examined the expression of arginine decarboxylase and agmatinase, the synthetic and degradative enzymes responsible for the synthesis and metabolism of agmatine following chronic delivery of anti-agmatine IgG given centrally.

Materials and Methods

Animals. Experimental subjects were Institute of Cancer Research (ICR) male mice (21-30 g, Harlan, Madison). Subjects were housed in groups of 8 in a temperature- and humidity-controlled environment and maintained on a 12 hr light/dark cycle with free access to food and water. These experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Chemicals. Agmatine sulfate, aminoguanidine, L-arginine, D-arginine, and NMDA were purchased from Sigma Chemical (St. Louis, MO). MK801 (Dizolcipine) was a gift of Merck. Endo-2 (YPPF) was synthesized by the University of Minnesota's Microchemical Facility. DAMGO ([D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin, mw 513.7) was purchased from Tocris Cookson (St. Louis, MO). All drugs were dissolved in 0.9% saline.

Anti-Agmatine antisera generation. Anti-sera to agmatine were raised in guinea pig as previously described in chapter 3.

Intracerebroventricular injection. All antibody- or saline-treated controls were administered i.c.v. in a 5 μ l volume in conscious mice according to the method of Haley and McCormick (Haley 1957). All injections were performed by one experimenter (KFK) who has over fifteen years of experience with the procedure.

The procedure for the injection was as follows: conscious mice were covered with a cloth to expose just the top of the head. The subject was then restrained at the base of the skull with the experimenter's thumb and forefinger so that the neck and jaw of the mouse were firmly, but gently, pressed against a firm flat level surface. A 50 μ l Hamilton syringe was

fitted to a 27-gauge needle with a rubber stopper positioned to expose 1.5 mm of the needle tip. The exposed tip was then inserted into the right lateral cerebral ventricle, through the scalp and the skull, 1 mm to the right of the skull's midline and level with the external auditory meatus; the skull was sufficiently soft to permit this insertion with minimal force. Once the needle was positioned, 5 μ l of solution was injected and the needle removed. This procedure takes less than a minute and requires no anesthetic, surgery, or incision.

Self-administration apparatus. Experimental chambers were Modular Mouse Test Chambers (Med-Associates, ENV-307CT, St. Albans, VT). Each chamber was housed in a sound-attenuating cubicle (Med-Associates, ENV-021M) and equipped with a 3.33 RPM syringe pump (Med-Associates, PHM-100) for drug delivery, 20 mg food pellet delivery system (Med-Associates, ENV-203-20), 2 ultra sensitive mouse levers (Med-Associates, ENV-310M) and 2 stimulus lights (Med-Associates, ENV-321M). A 4.8 W house light located at the top of the cage was illuminated during experimental sessions.

Behavioral procedure. The FR1 reward schedule coupled an active lever press with a delivery of 70 μ l of drug solution to the receptacle and illumination of the stimulus light directly above the lever. After each reward, there is a 5 second time-out period during which no reward was possible, regardless of additional lever presses (which was also recorded). Responding on the control lever resulted only in illumination of the stimulus light above it. Responses were monitored for both the active lever (the lever that drives delivery of fluid) and the control lever (the lever for which there was no associated reward provided in response to being pushed) and expressed as mean responses for each

test day. The control lever controls for random activity in the operant chamber during which the lever may be pushed. Each mouse was tested once daily (2 hour session) for the duration of the experiment.

Data analysis. The area under the curve (AUC) was determined by the trapezoidal rule using the statistical software package JMP[®] 6 from SAS. The resulting AUCs were analyzed using analysis of variance (ANOVA) from Prism 4.0. Significance was defined as $P < 0.05$.

ADC quantification. In a separate experiment, arginine decarboxylase was measured using rtPCR analysis. Mice were injected with either saline or anti-AG antibody i.c.v. every other day. Brain tissue was dissected at the following time points: 0, 2, 6, 10, 14 days so that mice were chronically exposed to the anti-AG antibody for 0-14 days. Tissue was flash frozen in liquid nitrogen following dissection and mRNA was analyzed using rtPCR quantification.

PCR analysis. Detection of ADC was done using standard procedures. RNA was isolated using a standard kit (RNAeasy Mini kit, Qiagen) followed by the reverse transcription of the mRNA into cDNA (QuantiTect, Qiagen). cDNA was generated as explained above, followed by rtPCR amplification using SYBR Green master mix (Qiagen, Valencia, CA). All qPCR reactions were performed and analyzed using the DNA Engine Opticon 2 (Bio-Rad Laboratories, Hercules, CA) and standardized to GAPDH. The critical cycle threshold was set at ≥ 10 standard deviations above baseline. PCR reactions for individual cDNA samples were performed in triplicate. The thermal cycling program included an initial denaturing step at 95°C for 15 min followed by 45 cycles consisting of

a 15 sec denaturing step at 94°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C. Following each extension, fluorescent intensity was measured at 75°C.

The primer sequences for ADC (GenBank accession number NM_172875, catalog number: QT00138012) were obtained from Qiagen (Valencia, CA) and yielded a predicted product size of 148 bp. The primers used for GAPDH (GenBank accession number NM_008084, catalog number: QT01658692) were also obtained from Qiagen (Valencia, CA) and yielded a predicted product size of 144 bp. PCR products were sequenced for verification.

Results

Agmatine sequestration attenuates oral fentanyl self-administration. We evaluated the effect of chronic administration of the anti-AG antibody (i.c.v) on oral self-administration of 10 mg/ml of fentanyl. The anti-AG antibody, normal guinea pig serum or saline was injected i.c.v. every other day until day 16 as described in the methods. Mice that received injections of normal guinea pig serum or saline developed increased active lever pressing for fentanyl over time distinct from that of control lever pressing, as expected (Fig. 1B and C). In contrast, sequestration of endogenous agmatine completely inhibited fentanyl self-administration, the active lever presses did not differ from the control lever presses in this condition (Fig. 1A). AUCs are represented in figure 1D.

Agmatine sequestration does not affect food-maintained responding. We next examined the effects of the anti-AG antibody on food-maintained responding to evaluate the possibility that the antibody treatment inhibited lever pressing in a non-specific manner. In contrast to the fentanyl self-administration experiment, neither sequestration

of agmatine through the anti-ag IgG or normal guinea pig serum had an effect on the acquisition of food-maintained responding; mice responded for food pellet delivery comparably in both conditions (Fig. 1E and F) with significant discrimination between the active and control levers.

Agmatine sequestration correlates with increased ADC expression.

Figure 2 illustrates an increase in the synthetic enzyme arginine decarboxylase on days 2 and 6. The increase in ADC returns to control levels at day 10.

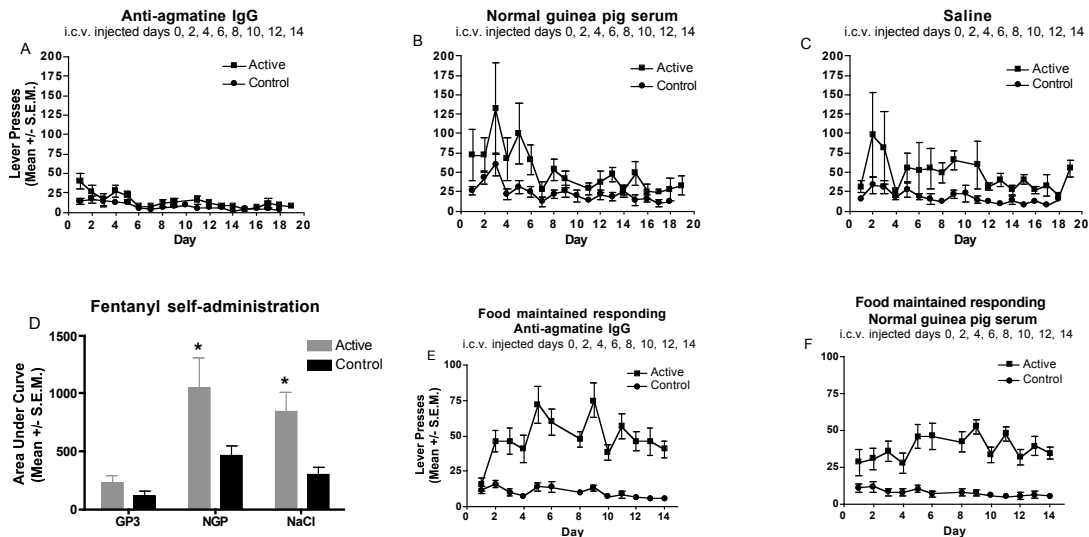


Figure 1. Anti-agmatine IgG I.C.V. in Fentanyl Self-Administration and Food-Maintained Responding: Fentanyl self-administration (upper panel) was compared in mice given the anti-agmatine IgG (A), normal guinea pig serum (B) or saline (C). In all panels the active lever that delivers fentanyl (squares) was compared to the control, or inactive, lever (circles). Fentanyl intake over the entire self-administration period is represented as area under the curve (AUC) (D) and was inhibited in mice with anti-agmatine IgG over a period of 18 days compared to both control subjects. Food-maintained responding was compared in mice given the anti-agmatine IgG (E) or normal guinea pig serum (F). Food-maintained responding was not affected. (*indicates significant difference in responding between the respective control and active lever within experimental group was determined by ANOVA, $p < 0.05$). $N=8$ per group

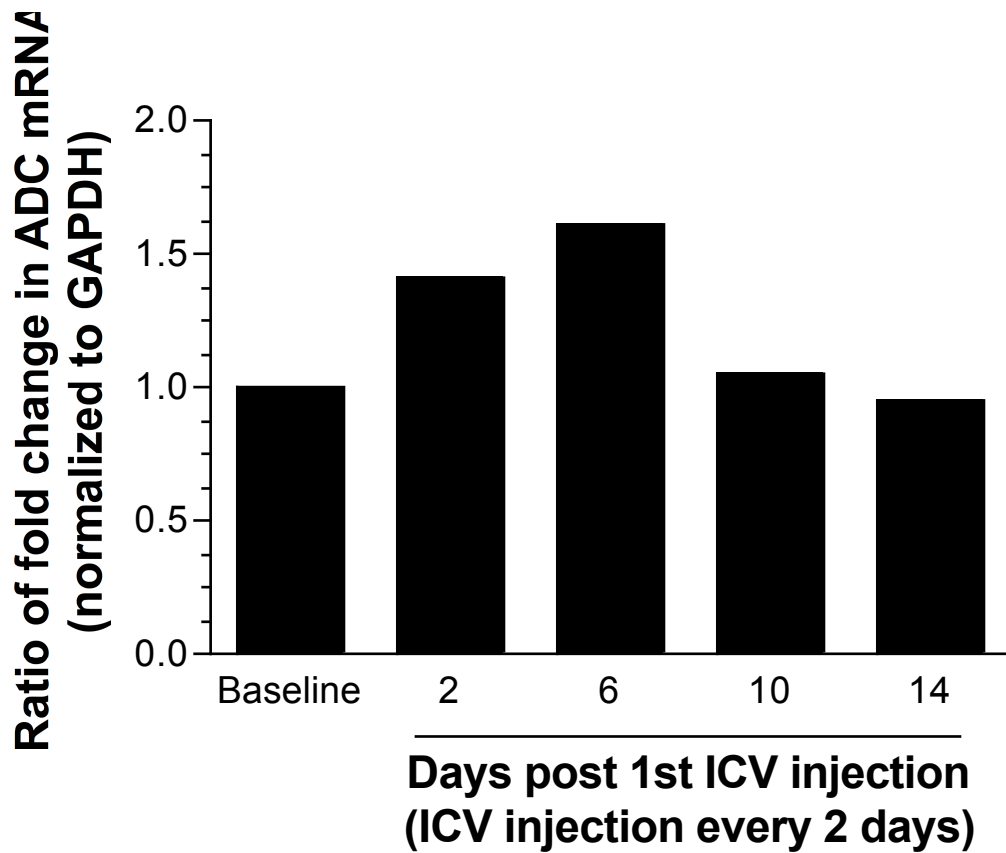


Figure 2. Anti-agmatine IgG I.C.V. arginine decarboxylase mRNA: Anti-agmatine IgG was injected ICV every other day for up to 14 days. Tissue samples were taken on days 2, 6, 10, 14 and analyzed for ADC mRNA using rtPCR. mRNA for ADC was upregulated on days 2 and 6 (40% and 60% respectively). mRNA levels returned to baseline by day 10.

Discussion

We have demonstrated that chronic sequestration of agmatine results in the same effects of exogenously applied agmatine in fentanyl self-administration. Because agmatine contributes to the reduction in fentanyl-induced tolerance as described in Chapter IV we hypothesized that the sequestration of endogenous agmatine would increase fentanyl self-administration. We then hypothesized that these paradoxical results might result from the difference in the time course of the antibody administration. To determine if chronic sequestration of endogenous agmatine results in compensatory production of agmatine we examined the relative mRNA levels of the synthetic and degradatory enzymes of agmatine. We found that in the early stages of the time course of chronic antibody administration mRNA levels for arginine decarboxylase were elevated suggesting that agmatine production is increased. Because the same concentration of the anti-AG IgG is given throughout the experiment the increased agmatine production might overcome the amount of agmatine that was sequestered by the antibody. Together these results show that endogenous agmatine regulates plasticity events such as opioid tolerance and addiction and that there is evidence for a compensatory mechanism, which turns on production of its synthetic enzyme, ADC.

Chapter VI

Summary and Conclusion

The last fifteen years represent an historic period in pain and addiction management. Experience with sustained release opioids suggests that scientific rationale, pharmaceutical development and governmental risk assessment may not predict unanticipated sociological response to introduction of a particular product. At the time that sustained release oxycodone was introduced to the market in 1995, the FDA and the manufacturer agreed upon the assertion that appropriate use of the product could reduce drug abuse risk (Cicero et al. 2005). At that time, what was known about the pharmacokinetics of opioids was that those with slow onset of action (resulting in delayed reinforcement) were thought to be less abused than rapidly released opioids (Cicero et al. 2005); therefore, the proposed reduced susceptibility to drug addiction for extended release opioids supported that claim of lower addiction liability. However, the impact of bypassing the sustained release mechanism was apparently not fully appreciated or considered; the result has been a significant increase in diversion of prescription medications for misuse concomitant with increased prescribing, although the pattern of source(s) of diversion are not entirely clear (Inciardi et al. 2009)

The more general outcome of the prescription drug abuse epidemic is that pain management as a whole is receiving increased revisitation at all levels (physician, pharmacist, patients) and increased restrictions on use of opioids for pain management are on the horizon. The Food and Drug Administration has been preparing guidelines for Risk Evaluation and Mitigation (REMS) (Craig 2010), which will require increased

training and certifications at the level of the physician, the pharmacist, and the patient. These social responses are largely in response to a phenomenon secondary to the medical experience of most of the patients and, as stated before, it is not clear at this time what the actual biological relationship between chronic pain and opioid addiction may be. As mentioned earlier, NIH has recognized the lack of information regarding this relationship and so developed increased interest and support for understanding the biological relationship between chronic pain and opioid addiction through support of the aforementioned RFA applications. The objective of this application was to develop a reliable model of prescription opioid self-administration that could be compared across established and recently introduced pain models. A second objective was to use this developed model to explore mechanisms that commonly govern opioid addiction and chronic pain, in this case the contribution of the NMDA/NOS cascade.

Chapter II examined fentanyl self-administration in distinct chronic pain conditions. We found that rates of fentanyl self-administration had distinct differences for each chronic pain condition we examined. Several other groups have looked at both inflammatory pain and neuropathic conditions in both a two-bottle choice paradigm and operant conditioning models. Our results support the conclusions from other research groups. Colpaert and colleagues studied a model of polyarthritis-induced inflammatory pain and showed that in a two-bottle choice model of fentanyl self-administration, rats in the pain group consumed more fentanyl than the non-arthritic rats, but less fluid overall. This increase in fentanyl consumption was attenuated following administration of dexamethasone, indicating that rats were self-administering fentanyl primarily for pain

relief and not extra-analgesic reward.

Martin and colleagues studied opioid self-administration following the onset of SNL-induced neuropathic pain and found that rats with neuropathic pain self-administered less heroin than their sham controls (Martin 2007) at lower concentrations of heroin (ascending limb), but at the higher concentrations of heroin, SNL mice responded more for heroin than their sham controls. These results are in agreement with the neuropathic pain results from our research program. The concentration we used for these studies was the same concentration that naïve mice responded the most for (10µg/ml) and we found that in both neuropathic pain groups (SNL- and vincristine-induced neuropathic pain) mice responded less than their respective controls for fentanyl. It is possible that mice in a chronic hyperalgesic state may respond to concentrations of fentanyl that are not rewarding in naïve or control animals. Martin et al., 2007 also examined self-administration following clonidine administration and found that following clonidine injection rats with SNL-induced neuropathic pain self-administered less heroin, while the sham control rats did not change their self-administration rates. Together, these results indicate that opioid consumption may not be rewarding for the subjects with pain.

It is interesting to note that mice with idiopathic pain originating as a result of sickle-cell anemia self-administered more fentanyl than the control group. This result indicates that fentanyl self-administration may be used as a tool for investigating discrete differences in pain groups when pain is of an idiopathic nature.

Several research programs could be developed to further evaluate the differences in self-administration rates among the different pain groups. To date there is not a

comprehensive study examining the effects of rescue analgesia on self-administration rates between either different pain types or analgesics. In addition, studies have been published suggesting that reward centers of the brain are differentially affected following the onset of pain. It has been found that following spinal nerve ligation dopamine release is altered from the VTA to the nucleus accumbens (Narita et al. 2004; Niikura et al. 2008), but it has not been studied if these effects extend to other forms of chronic pain and if chronic pain may actually protect against addiction in this manner. It is also thought that changes in input to the VTA from the basolateral amygdala and the central nucleus of the amygdala, areas which are both thought to be responsible for the negative emotional aspects of addiction and pain occur under conditions of chronic pain (Besson 1999; Price 2000). The effects on the VTA have not been studied during opioid self-administration.

The remainder of the research program focused on the role of agmatine in neuroplasticity events of opioid self-administration and opioid-induced tolerance. Chapter III examined the role of agmatine in fentanyl self-administration through exogenous administration. We found that agmatine inhibited fentanyl self-administration when injected i.c.v. and that the inhibition appears to be drug specific as agmatine had no effect on food self-administration. To extend the originally published study we evaluated this effect in a reinstatement model of self-administration and found that administration of agmatine also resulted in a trend towards decreased fentanyl self-administration during the reinstatement period.

Agmatine is synthesized by arginine decarboxylase and degraded by agmatinase; it has been shown to have temperature dependent be released from nerve terminals upon calcium dependent depolarization. One other criterion for classification as a neurotransmitter is the effects from exogenously administered agmatine must mimic the effects of endogenous agmatine. To further evaluate this criterion we sought to examine endogenous agmatine in acute opioid tolerance as described in Chapter IV. In order to evaluate the presence of an endogenous antagonist we used an antibody targeted to agmatine to sequester agmatine in the brain. We expected that the absence of endogenous agmatine would produce effects opposite of exogenously administered agmatine. Previously, we have shown that exogenously administered agmatine prevents acute morphine tolerance, we then expected that when pretreated with the anti-agmatine antibody tolerance would be exaggerated or induced following a low dose of morphine. In fact, the results reflected the anticipated outcome. This result describes the role of an endogenous NMDAR antagonist as neuroprotective against morphine tolerance.

Finally, Chapter V describes the effect of chronic sequestration of endogenous agmatine in opioid self-administration. We found that mice injected with the anti-agmatine antibody did not acquire fentanyl self-administration behaviors, similar to mice that had been injected with agmatine in Chapter III. This paradoxical result prompted us to evaluate the levels of agmatine's synthetic enzyme, ADC and degradative enzyme, agmatinase. Although there was no change in agmatinase levels (data not shown), we found that ADC levels were increased at days 2-6, which is a critical time in acquisition of fentanyl self-administration behaviors as shown in Chapter III. Memantine, another

NMDA receptor antagonist has also been shown to have similar effects on opioid tolerance and self-administration (Harris et al. 2008; Kuzmin et al. 2008; Mendez and Trujillo 2008; Aguilar et al. 2009).

Future Directions

To further characterize the role of agmatine, it is necessary to evaluate NMDA receptor binding. We have developed a method to target NMDA subunits using siRNA directed at NR2B and have preliminary results suggesting that the NR2B subunit is necessary for agmatine's actions. It is also important to characterize the mechanism by which ADC is upregulated in neuroplasticity. The upregulation of ADC upon the functional absence of agmatine suggests that endogenous agmatine has an important neuroprotective and regulatory role in the CNS.

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Carrie Wade
Graduate Program in Pharmacology
6-120 Jackson Hall
321 Church st SE
Minneapolis, MN 55455
Tel: 612.625.1106
Fax: 612.625.8408
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