ALLEVIATION OF CHRONIC NEUROPATHIC PAIN BY AGMATINE REQUIRES THE GLUN2B SUBUNIT OF THE NMDA RECEPTOR

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Dedication

To Dad. Thank you for valuing education and
encouraging me year after year.
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Chapter 1: Introduction

Synopsis

In 2017, the United States remains in the midst of what has been named the Opioid Epidemic, characterized by drug overdoses surpassing automobile accidents as the leading cause of death with a majority of these overdose deaths involving opioids (Wilson-Poe and Morón 2017). The Center for Disease Control and Prevention (CDC) responded to the rise of opioid abuse, overdose, addiction, and misuse by writing and releasing a set of opioid prescribing guidelines for the treatment of chronic non-cancer pain (Van Demark, Chang and Heinemann 2016). However, these prescribing guidelines present challenges to physicians and patients seeking to maintain the use of opioids in a safe and controlled manner, leaving patients with few analgesic options to control chronic pain (Kroenke and Cheville 2017). While the solution for this situation must be multi-faceted, the work presented in this thesis will focus on an understanding of the development and maintenance of chronic neuropathic pain and effective non-opioid analgesics.

Prevalence of Pain

Pain, as defined by the International Association for the Study of Pain (IASP), is an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (1986)." Estimates of pain in America are as high as 126.1 million adults experiencing

some form of pain within the previous three months, and 25.3 million adults suffering from chronic pain, while 23.4 million experiences a large amount of pain (Nahin 2015). Patients with the highest levels of pain use more health care, suffer more disability, and have a worse health status than those with lesser or no pain. Internationally, the rates of chronic pain in developed countries (37.3%) and developing countries (41.1%) demonstrate the vast population of chronic pain patients (Tsang et al. 2008). Pain presents in higher rates among women and the elderly and is associated with depression-anxiety spectrum disorders, but the majority of individuals with self-reported chronic pain do not qualify as presenting with depression or anxiety disorders (Tsang et al. 2008).

Pain of Neuropathic Origin

Neuropathic pain is a pain class characterized by lesion or dysfunction of the central nervous system (CNS) from direct nerve or spinal cord injury, originating from diseases such as diabetes, stroke, or multiple sclerosis (Colombo, Annovazzi and Comi 2006). While it remains clinically difficult to classify and evaluate pain etiology in patients (Steingrímsdóttir et al. 2017), around 9.8% of the population of pain patients suffer from pain of neuropathic origin (Yawn et al. 2009).

Pain of neuropathic origin is traditionally thought to be responsive to anticonvulsant therapy (Tremont-Lukats, Megeff and Backonja 2000, BLOM 1962), with gabapentin, carbamazepine and phenytoin showing efficacy at

relieving diabetic neuropathy and trigeminal neuralgia (Jensen and Larson 2001, Jensen 2002). Opioids are accepted as a class of treatments for neuropathic pain as long as an acceptable side-effect profile is maintained (Colombo et al. 2006). Mu opioid receptor (MOR) agonist medications (Jones, Lawson and Backonja 2016) are thought to be effective in human neuropathic pain when given in sufficient doses and/or in combination with adjuvants (Varrassi et al. 2009). However, their long-term use is still debated (Cooper et al. 2017) due to small trial sizes, small effect sizes, and a lack of well-controlled studies. Recent meta-analyses (Derry et al. 2016, Gaskell et al. 2016, Stannard et al. 2016) have concluded that the evidence for opioid effectiveness under conditions of neuropathic pain is either moderately supportive or inconclusive due to the broad heterogeneity of neuropathic conditions comprising the clinical trials. Placebo-controlled clinical trials of opioids in neuropathic pain are ethically contraindicated as a placebo arm would experience unrelieved neuropathic pain. This leads to innovative trial design such as of several doses of the same opioid therapy rather than a placebo control (Rowbotham et al. 2003).

Animal Modeling of Chronic Pain

Human epidemiological studies aimed at understanding chronic pain face the challenge of concretely defining chronic pain. A meta-analysis found that among 86 studies purporting to evaluate chronic pain, no studies employed exactly the same criteria to their sample population (Steingrímsdóttir et al. 2017).

A benefit of conducting chronic pain research in animal models is the ability to precisely control the nature of pain and experimental interventions applied (Mogil 2009). These studies are essential to finding and characterizing novel therapeutics capable of addressing the lack of safe and effective pain therapeutics (Barrett 2015). Animal models are especially beneficial when evaluating therapeutics intended to be given on a chronic schedule at determining the safety and early stage toxicity of new chemical entities (McGonigle and Ruggeri 2014). In an effort to directly compare the results across all of the studies presented in this thesis, I have used a well-characterized and extensively used model of chronic neuropathic pain as developed by Decosterd and Woolf (Decosterd and Woolf 2000) in all of the pain studies presented in this thesis in an effort to tightly control the model, allowing for direct comparisons between each pharmacological tool used.

NMDA Modulation to Alleviate Neuropathic Pain

N-methyl-D-aspartate receptor (NMDAR) antagonism has long been investigated as a therapeutic avenue in the treatment and control of neuropathic pain in both human- and animal-based studies (Sang 2000, Collins et al. 2010, Aiyer et al. 2017) due to this receptor's role in persistent pain pathways (Bleakman, Alt and Nisenbaum 2006) including pain arising from damage to peripheral or central nerves. Glutamatergic signaling is altered in multiple ways along nociceptive pathways in chronic pain states including an increase in the

release of glutamate in the dorsal horn of the spinal cord from primary afferent terminals (Chen et al. 2009), a decrease in the clearance rate of glutamate from the synaptic cleft (Sung, Lim and Mao 2003), and an increase in the number of NMDA receptors (Salter and Pitcher 2012). A plethora of NMDAR antagonists have been studied in clinical trials of chronic neuropathic pain including ketamine, methadone, memantine, amantadine, dextromethorphan, carbamazepine, phenytoin, and valproic acid which represent action at allosteric binding sites, subtypes 2A through 2D, non-competitive binding, and pre-synaptic NMDA inhibition of glutamate release (Aiyer et al. 2017). The most consistently efficacious therapeutic NMDA antagonist, intravenous ketamine, is not FDA approved for the treatment of neuropathic pain conditions, and drug-induced liver injury or altered liver function has been reported in patients receiving ketamine treatment (Noppers et al. 2011).

While NMDA antagonism is an attractive pharmacological approach, physiological NMDA activity is essential for the continuity of normal function. As such, pharmacological agents that inhibit broad spectrum NMDA activity have impactful clinical side effects including psychotic symptoms, memory impairment, and motor impairment (Olney 1994, Lipton 2004, Neznanova et al. 2000). These findings have led to the search for an NMDA antagonist that preferentially and site-selectively inhibits excessive NMDA activity without disrupting normal, physiological function.

Agmatine: A Novel NMDA Antagonist

Agmatine was first purified from herring sperm and characterized by Noble Laureate Albrecht Kossel in 1910 (Kossel 1910), but was only investigated sparingly until 1994 (Li et al. 1994) due to the erroneous assumption that agmatine was not synthesized in mammals (Tabor and Tabor 1984). Agmatine is synthesized by arginine decarboxylase (ADC) in both mammalian and plant tissue; the sequence of human ADC is distinct from other forms (Zhu et al. 2004). The degradation of agmatine to putrescine and urea occurs mainly via agmatinase-mediated hydrolysis (Sastre et al. 1996). ADC and agmatinease respectively constitute the main synthetic and degradative pathways for endogenous agmatine.

Early studies of agmatine elucidated its efficacy at antagonizing NMDARs in cultured rat hippocampal neurons (Yang and Reis 1999). Putrescine was ineffective at reproducing this block, suggesting that the guanidine is the active moiety required for agmatine's block of NMDAR. We have recently expanded this line of investigation to interrogate the subunit requirements for agmatine's inhibition of NMDAR currents through the use of GluN2B-floxed mice and whole cell patch clamp electrophysiology (Wataaja, in preparation). This area of research will be discussed in depth in Chapter 4 of this thesis.

Functionally, agmatine has demonstrated efficacy at and inhibiting glutamate-induced neurotoxicity through NMDA antagonism in cultured cerebellar

granule cells (Olmos et al. 1999). *In vivo,* many studies have demonstrated agmatine's efficacy at either direct analgesia in models of neuropathic pain (Karadag et al. 2003, Kotagale et al. 2013, Fairbanks et al. 2000) or as an adjunct to modulate opioid analgesia (Kolesnikov, Jain and Pasternak 1996, Su, Li and Qin 2003, Regunathan 2006). The foundation of these studies indicates that agmatine is a viable candidate for the analgesic relief of chronic, neuropathic pain but more work is needed to understand it's side effect profile and it's efficacy as compared to clinically relevant analgesics. This work will be presented in Chapter 2.

AAV Gene Therapy to Deliver Chronic Therapeutics

Gene therapy is an emerging strategy for providing long-term relief for chronic pain syndromes (Guedon et al. 2015). The adeno-associated viral vector offers very desirable traits for application to gene therapy, and recently became the first vector system approved for clinical applications (Bryant et al. 2013). The parent virons do not replicate and are considered to be non-pathogenic, reducing the possibility of a severe immune reaction that could be harmful or deadly for a patient (Mingozzi and Buning 2015). This family of vectors also has an extended period of expression, having been seen to express for up to a decade (Buchlis et al. 2012). This feature makes them desirable to apply to a disease state such as neuropathic pain that requires continuous modulation. AAV vectors also come in a variety of serotypes with different patterns of expression. Interestingly, only a

small subset of these serotypes are able to cross the BBB (Zincarelli et al. 2008). It has also been noted that the route of delivery of the vector can have an impact on the pattern of expression (Thierry et al. 1995). For these reasons, AAV therapy provides the ability to customize expression to areas of interest via multiple strategies (Mingozzi and Buning 2015). In Chapter 3, I will describe our use of two AAV serotypes, AAV5 and AAV9, to deliver arginine decarboxylase to the central nervous system, the subsequent increase in endogenous agmatine, and the attenuation of neuropathic pain behaviors.

AAV Targeting via Differing Serotype Expression

All AAV serotypes have the same genome size and organization. They are all single-stranded DNA parvoviruses of the genus Dependovirus. Twelve human serotypes have been discovered (Daya and Berns 2008), numbered AAV1-AAV12. The serotype of each AAV vector will have an impact on where the vector expresses. The injection route, as well as the model used, will also show different patterns of expression. The following sections highlight the differential expression achieved through different AAV serotypes, as well as the injection route used to achieve that pattern of expression. This information is critical for the design and implementation of targeted gene therapy to decrease off-target effects.

AAV2

AAV2 was the first serotype ever utilized for gene transfer with initial publication in 1984 (Hermonat and Muzyczka 1984). In a systematic analysis of AAV serotypes 1-9 in a mouse model utilizing tail vein injection, Rabinowitz and colleagues characterized AAV2 as having low expression and slow onset of expression (Zincarelli et al. 2008). In a more recent analysis utilizing mouse as well as marmoset and macaque models, AAV2 was found to have a distinct pattern of expression compared to 1, 5, 8, and 9 (Watakabe et al. 2014). While all serotypes were able to transduce cortical cells in the marmoset cerebral cortex, AAV2 had a noticeably smaller spread and a neural tropism as compared to the robust glial expression of the other serotypes, with these results being confirmed in the mouse and macaque models.

AAV2 does not cross the BBB and enter the CNS following facial vein injection in a neonatal mouse (Zhang et al. 2011). A team of researchers, however, was able to develop a technique of inducing opening of the BBB by ultrasound and subsequently showed activity of the AAV2 virus in the rat brain following peripheral delivery(Alonso et al. 2014). A consideration needs to be made from a therapeutic and clinical standpoint whether it would be more beneficial for the patient to undergo this stimulation in order to open the BBB and allow the vector to enter the CNS or if the therapy should be developed with a

different serotype that can cross the barrier naturally if the area to be targeted is within the CNS.

AAV5

AAV5 is characterized by low expression as well as a slow onset compared to vectors 1-4 and 6-9 after tail vein injection in mail mice (Zincarelli et al. 2008). Under a general CMV promoter in a marmoset cerebral cortex model, as well as mouse and macaque, AAV5 was found to transduce mostly glial cells but was able to transduce neurons when placed under a CaMKII promoter (Watakabe et al. 2014).

In study aimed at elucidating various vector's ability to cross the BBB, it was noted that AAV5 did not enter the CNS following a facial vein injection of virus in a neonatal mouse (Zhang et al. 2011). However, when delivered intrathecally, AAV5 was able to transduce a wide subset of the CNS, including hindbrain, isolated neurons and astrocytes in the midbrain, as well as rostrally to the olfactory bulb. This pattern of expression potentially indicates that AAV5 is able to spread through the movement of cerebrospinal fluid (CSF) if delivered within the CNS (Schuster et al. 2014a). The use of intrathecally delivered AAV5 is further discussed in Chapter 3.

AAV9

AAV9 contains the inherent ability to pass through the BBB, a traditionally difficult to penetrate barrier (Byrne et al. 2015). Before that, it was known that the

capsid had a high affinity for the heart following IV injection in adult mice (Pacak et al. 2008). While AAV2 and AAV5 were found to have low expression in a mouse model following tail vein injection, AAV9 was characterized to have both a rapid onset of expression as well as high expression as determined by bioluminescence imaging (Zincarelli et al. 2008).

Additionally, systematic studies of AAV9 show that its efficacy at crossing the BBB is in part determined by the age of the subject and the stage of maturation of their BBB. Following IV injection of AAV9 at various ages, it was seen that the resulting expression was more efficient in neonatal than adult mice (Foust et al. 2009). Neonatal mice showed extensive transduction of neurons throughout the brain whereas adult mice showed transduction limited to mostly astrocytes and small proportion of neurons.

It is important to consider the route of administration when evaluating distribution of AAV9, despite its ability to cross the BBB. In a study directly comparing intravenous and intrathecal injection in a mouse model of AAV9, it was noted that intrathecal delivery of vector resulted in increased efficiency of transducing sensory neurons and the central nervous system (CNS) (Schuster et al. 2014b). The vector delivered intravenously was able to cross the BBB and express in a selection of isolated neurons and astrocytes, but intrathecal delivery resulted in a more intense and more widespread distribution. Also of note from this study was the observation that intrathecal delivery of AAV9 vector resulted in transduction of peripheral tissues, signifying that the vector was able to

redistribute into the systemic circulation from the subarachnoid space. Intrathecal AAV9 is further discussed in Chapter 3.

AAV Promoter and Enhancer Sequences

In the previous section describing serotype, a majority of the studies were conducted with vectors containing a cytomegalovirus (CMV) promoter. This promoter is commonly used as a general promoter/enhancer of expression with the thought that it will provide a robust and long-lasting expression, (McCown et al. 1996). More recently, studies have been published characterizing the expression and distribution of this promoter compared to other general promoters as well as limited promoters (for example, neuron-specific). The following sections will focus on these different promoter/enhancer sequences.

Constitutive CMV Promoter

The cytomegalovirus (CMV) promoter is the most widely used promoter in viral vector design, as it leads to a relatively stable expression and broad distribution. This was systematically tested by Damdindorj et al. (Damdindorj et al. 2014) in a study comparing CMV to 5 other constitutive promoters in a variety of cell lines. In this study, they observed that the CMV promoter drove the highest level of GFP expression across the different cell lines and maintained expression for the 8 weeks of the study. However, the same study found that using a CMV promoter to drive gene editing led to the lowest efficiency among

the compared promoters. It is important to note that this study was conducted using all cancerous cell lines, and that further study in either primary culture or animal models would be beneficial.

Further advancements in constitutive promoter design have been suggested and designed, with hybrid CMV-based promoters gaining interest in the field (Fitzsimons, Bland and During 2002). These modifications are intended to enhance gene expression as well as stabilize gene expression, hopefully making gene therapy in chronic diseases like chronic neuropathic pain a more viable option as expression was seen for up to a year.

CMV can also be used as an enhancer, and has traditionally been combined with a chicken-beta-actin promoter (Xu et al. 2001). In one initial study, this CMV-beta-actin combination resulted in an 137-fold increase in expression compared to a CMV promoter/enhancer construct when studied in neonatal mice. Another study utilizing this promoter/enhancer combination showed a rapid and robust response in a mouse model of lysosomal storage disease, another disease requiring extended therapy (Daly et al. 1999). The injection of vector occurred in neonatal mice and was detected 2 weeks after birth, and no reduction in expression was noted for 16 weeks.

Additional Constitutive Promoters

While a study comparing the CMV promoter to various constitutive promoters found CMV to have the highest level of GFP expression, CMV was not

the most efficient at driving gene editing, a common application for AAV gene therapy. The constitutive promoters human beta-actin (hACTB) and human elongation factor-1alpha (hEF1a) were especially notable in the DLD-1 cell line (Damdindorj et al. 2014). In the previously mentioned study comparing CMV-beta-actin to CMV alone, elongation factor 1-alpha (EF1-alpha) was also analyzed. It was seen that while the EF1-alpha promoter had higher expression than CMV alone *in vivo*, CMV-beta-actin was significantly higher than both of the previous constructs (Xu et al. 2001).

Neuron-Specific Promoters

It is common that a therapy is only needed in neuronal cells, creating the needs for neuron-specific expression. Human synapsin 1 (hSYN1) can be employed for this purpose. It has been seen that an AAV9 serotype in combination with an hSYN1 promoter achieves widespread, due to the tropism of AAV9, neuron-specific expression following intracerebroventricular (i.c.v.) delivery in neonatal mice (McLean et al. 2014). In a similar study conducted in a rat model, this neuron-specific expression was seen to be long-term if the vector was given at the appropriate dose (Kugler, Kilic and Bahr 2003). This study compared hSYN1 to another neuron-specific promoter, namely neuron-specific enolase (NSE). They found that NSE was not completely neuron-specific but rather also expressed in glial cells. It also had a lower level of transcriptional targeting compared to hSYN1.

A third neuron-specific promoter, calcium/calmodulin-dependent protein kinase II (CaMKII), has been shown to transduce inhibitory somatosensory cortical neurons in a mouse model when packaged with AAV2 (Nathanson et al. 2009). It is important to note that virus was directly injected into the somatosensory cortex for this experiment, as AAV2 is not expected to cross the BBB. However, in a study utilizing a marmoset model, CaMKII was delivered also to the somatosensory area using serotypes 1, 2, 5, 8, and 9. This study also injected virus into both male and female mice, as well as macaque. This second study examining CaMKII did not observe the selection for inhibitory somatosensory cortical neurons but rather saw that a majority of neurons were transduced (Watakabe et al. 2014).

Thesis Preview

Based on this scientific foundation, I sought to determine the efficacy and potential side effects of exogenous (small molecule) and endogenous (viral vector) approaches to delivering agmatine in a model of chronic neuropathic pain. I sought to determine if an increase of agmatine would prevent or reverse the development of hyperalgesia in a spared nerve injury model of neuropathic pain, as well as evaluate motor function and other behaviors to support translation of agmatine into a clinical setting. Finally, I sought to evaluate agmatine's signaling pathway using a GluN2B-floxed conditional knockout mouse.

Thesis Objectives

Effective treatment for chronic pain patients remains an area of largely unmet need. However, chronic pain patients receiving traditional opioid therapy are consistently surrounded by the potential risks and social stigmas of opioid dependence, misuse, and addiction. These concerns are heightened in the face of the expanding opioid epidemic. The need for new, non-opioidergic therapeutics for management of the large population of chronic patients is widely recognized. Agmatine, also known as decarboxylated arginine, is an endogenous small molecule that has been shown to modulate maladaptive neuroplasticity that underlies the experience of chronic pain. Agmatine has been established to meet the criteria of acting as a neurotransmitter including synthesis in neurons (Wang et al. 2014), release from nerve terminals (Goracke-Postle et al. 2007), and binding to post-synaptic receptors (Gibson et al. 2002, Yang and Reis 1999). We have previously demonstrated the efficacy of exogenously delivered agmatine in reversing chronic pain behaviors in models of neuropathic pain. Targeting primary sensory neurons through gene vectors such as serotypes of the adeno-associated virus has recently been identified as a powerful emerging strategy to treat chronic, intractable pain (Pleticha, Maus and Beutler 2016). Gene therapy has been approved for market use in Europe and the United States, making it a viable tool for translation from bench side to clinic. To this end, a viral vector encoding the synthetic enzyme for agmatine, namely arginine decarboxylase was developed. It has been shown that intrathecally injected viral vector particles

distribute to sites of interest for chronic pain (Vulchanova et al. 2010, Schuster et al. 2014a, Schuster et al. 2014b).

The *primary objective* of my thesis work has been to expand both the application and mechanistic understanding of agmatine as a non-opioidergic therapeutic in the treatment of chronic pain. The *central hypothesis* of this work is that enhanced expression of arginine decarboxylase in nociceptive pathways results in long-term reduction of neuropathic pain due to agmatine production and agmatine's antagonism of the NMDA receptor. The *rationale* for this research was that delivery of a gene therapy to enhance agmatine's inhibition of NMDA signaling would be a viable, long term solution for management of chronic pain. Through the next sections I will expand upon the dual public health crises of chronic pain and prescription opioid abuse. These call for new, non-opioid therapeutic approaches for chronic pain, leading to the therapeutic development of agmatine as an NMDA receptor antagonist.

Chapter 2

In this thesis, I will report my work utilizing these ideas of NMDA modulation via agmatine. In Chapter 2, I present a set of experiments intended to pursue Specific Aim 1:

Specific Aim 1: Compare the pharmacological effects of agmatine to its primary metabolite and gold standard NMDA receptor antagonists.

The experiments developed to address Specific Aim 1 assessed the effects of NMDA antagonism in chronic neuropathic pain by agmatine, its primary metabolite putrescine, and several gold standard NMDA receptor antagonists. These comparative studies were essential to expand upon the prior knowledge contributed by our research group regarding the effects of exogenously delivered agmatine on chronic neuropathic pain. Specifically, we sought to understand the effects of agmatine compared to its primary metabolite, putrescine, the primary gold standard non-selective NMDA receptor antagonist, MK-801, and the primary gold-standard GluN2B subunit-selective NMDA receptor antagonist, ifenprodil. Both prevention of the development of neuropathic pain as well as the reversal of established pain by agmatine is presented. Additionally, we were able to define an optimal drug delivery schedule of agmatine as well as determine the effectiveness of agmatine treatment in the maintenance phase of established neuropathic pain. These experiments provided essential foundation which informed and enabled the pursuit of Specific Aims 2A, 2B, and 3 of the thesis proposal.

Chapter 3

Based on our preliminary data, I hypothesized that delivery of an adeno-associated viral vector that overexpresses the enzyme for conversion of L-arginine to agmatine would be effective at preventing or reversing chronic

neuropathic pain. This hypothesis was addressed through delivery of a vector to overexpress the synthetic enzyme for agmatine in a rodent model of neuropathic pain. Tactile sensory and motor function behavioral testing was performed as well as immunohistochemical assessment to demonstrate anatomical distribution of a reporter gene (green fluorescent protein) throughout the central nervous system.

In Chapter 3, I present extensive behavioral characterization of the use of agmatine-based gene therapy to prevent or reverse chronic neuropathic pain in both rat and mouse models. These experiments were designed to address Specific Aim 2A:

Specific Aim 2A: Determine the ability of overexpression of arginine decarboxylase to prevent and reverse chronic pain behaviors

The experiments developed to address Specific Aim 2A assessed the effects of intrathecal delivery of an adeno-associated viral vector that carried the gene for arginine decarboxylase on neuropathic pain. We observed that in both rat and mouse, pre-treatment with this viral vector construct reversed neuropathic pain. We also observed that post-treatment of the viral vector also reversed neuropathic pain in mice. We used an immunoneutralization approach to demonstrate that both pharmacological outcomes are dependent on spinal agmatine.

Based on our preliminary data collected from pursuit of Specific Aim 2A, I also hypothesized that delivery of this same AAV vector designed to express arginine decarboxylase (hADC, the synthetic enzyme for agmatine) would be effective at reversing and preventing the development of chronic pain with efficacy lasting *up to a year following injection*. I pursued a second aim to address this hypothesis:

Specific Aim 2B: Determine the duration and therapeutic window of AAV-hADC treatment

To assess the duration of AAV-hADC mediated analgesic efficacy after nerve injury, we studied various cohorts of mice for varying durations of time out to a year post-injury. To assess the therapeutic window of efficacy after nerve injury, we studied various cohorts of mice that were injected with AAV-hADC either two weeks after nerve injury or as late as nine months after nerve injury. These experiments demonstrated that the duration of action of our gene therapeutic as well as the window of efficacy after injury where the therapeutic is still effective at reversing behavioral hypersensitivity is as late as nine months post-injury and at least 11 post-injection.

Chapter 4

In Chapter 4, I evaluated a potential signaling pathway for agmatine by utilizing a conditional genetic knockdown of the GluN2B subunit of the NMDA receptor. This line of research emerged from the work represented in Chapter 2 and 3 and previous literature. Our working hypothesis emerged that agmatine requires the GluN2B subunit of the NMDA receptor for reversal of behavioral hyperalgesia. To address this hypothesis, I pursued the following Specific Aim:

Specific Aim 3: Determine the NMDA receptor subunit target of agmatine in vivo.

I addressed this hypothesis by delivering exogenous agmatine to either transgenic GluN2B-deficient or wildtype mice under conditions of nerve injury and evaluated the resulting anti-hyperalgesia. In Chapter 4, I describe our development of the knockdown, as well as behavioral data examining agmatine's efficacy in both knockdown and wildtype animals in models of chronic neuropathic pain as well as opioid tolerance.

Summary

After conducting my thesis research, I have completed several key objectives. I have evaluated the behavioral impact of exogenous delivery of agmatine and various NMDA inhibitors in chronic neuropathic pain. Additionally, I

have systematically evaluated the impact of AAV gene therapy delivering the synthetic enzyme for agmatine, arginine decarboxylase, in a model of chronic neuropathic pain. Lastly, I evaluated the necessity for the GluN2B subunit of the NMDA receptor for agmatine's efficacy in reversing hyperalgesia in a model of chronic neuropathic pain as well as preventing the development of morphine tolerance.

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Chapter 2: Prevention and Reversal of Chronic Neuropathic Pain via Agmatine

Synopsis

Agmatine, decarboxylated L-arginine, is endogenously synthesized by arginine decarboxylase and has shown to effectively inhibit expression of neuropathic and inflammatory pain when delivered either centrally or systemically. In this chapter, I will show pharmacological and behavioral confirmation of agmatine's efficacy in preventing and reversing both evoked and non-evoked manifestations of neuropathic pain, as well as a direct comparison to the clinically used standard of analgesia, morphine. These data support the hypothesis that agmatine is an effective analgesic and a viable candidate for clinical translation.

(C.P planned and conducted the experiments and behavioral analysis. K.K. conducted experiments. C.A.F and G.W. contributed to analysis and editing.)

Introduction

Agmatine was first discovered in 1910 by the Nobel laureate Albrecht Kossel (Kossel 1910). Agmatine, the decarboxylated form of L-arginine, is endogenously synthesized in mammals by arginine decarboxylase (Li et al. 1994). Agmatine meets many of the criteria of acting as a neurotransmitter/neuromodulator including synthesis in neurons (Wang et al. 2014), release from nerve terminals (Goracke-Postle et al. 2007), and binding to post-synaptic receptors (Gibson et al. 2002, Yang and Reis 1999). This small molecule is an inhibitor of the N-methyl-D-aspartate receptor (Yang and Reis 1999), a receptor found on nociceptive neurons that relay pain signals from the periphery, through the spinal cord and into the brain for sensory processing. This action through the NMDA receptor rather than traditional opioid receptors makes agmatine a viable and promising therapeutic target, allowing for modulation of pain sensation without established concerns surrounding the opioidergic system (Fairbanks et al. 2000). In this chapter, I will assess agmatine's efficacy, duration of action, and role in maladaptive neuroplasticity prior to or following the development of peripheral neuropathic pain.

Spared Nerve Injury Model of Neuropathic Pain

Neuropathic pain is an umbrella term used to describe pain originating from lesion or dysfunction of normal sensory pathways (Jensen et al. 2001). This dysfunction may arise from disorders such as diabetes, malignant diseases,

physical trauma, vitamin deficiencies, and immune deficiencies (Woolf and Mannion 1999). Clinically, neuropathic pain is characterized by both spontaneous pain as well as stimulus-evoked pain (Marchettini et al. 2006). We have chosen the spared nerve injury (SNI) model of rodent neuropathic pain (Decosterd and Woolf 2000) to represent these clinical symptoms. In this model, the sciatic nerve is exposed and its three branches (tibial, peroneal, and sural) are identified. Both the tibial and peroneal branches are ligated and cut while the sural branch is spared. This ligation results in a marked increase in mechanical hypersensitivity in the lateral portion of the paw, the area innervated by the sural nerve. Induction of hypersensitivity occurs within the first day post-surgery and results in a long-lasting hypersensitivity that is not likely to resolve. Additionally, all animals that receive this surgery develop the pain phenotype, indicating a robust model.

Morphine's Efficacy in Neuropathic Pain

Morphine remains one of the most studied and characterized clinically-available analgesics (Corbett et al. 2006), and is listed by the World Health Organization as an essential medicine for post-operative, analgesic and palliative care (Organization 2011). While morphine has clinically shown efficacy for short term reversal of pain of neuropathic origin (Wang et al. 2017), recent analysis has shown little to no support for the claim that morphine given alone is an effective chronic treatment for chronic, neuropathic pain (Cooper et al. 2017a),

with some studies even reporting a trend towards a decrease in physical function and an increase in disability following chronic morphine use (Bostick et al. 2015). However, many pre-clinical studies using animal models of chronic, neuropathic pain continue to report efficacy of morphine given alone in reversing pain behaviors (Smith et al. 2017). Divergence between these findings may be due in part to the well-documented phenomena of opioid tolerance (decreased efficacy following repeated administration of the same drug) following chronic morphine treatment (Wilson-Poe, Jeong and Vaughan 2017, Elhabazi et al. 2014, Dighe et al. 2009). In this chapter, I have included a control of centrally delivered morphine in early and late phase neuropathic pain in order to compare the efficacy, time to onset, and duration of effect of the well characterized morphine to agmatine, our experimental treatment.

Evoked vs. Constitutive Pain

Evoked (reflexive) measures of pain behavior remain one of the most commonly used indicators of the presence and level of neuropathic pain in animal models. These assays evaluate the level of behavioral pain responses following heat, cold, electrical or mechanical stimuli (Gregory et al. 2013). While these tools remain useful, a major advancement in pain research has been the understanding of a variety of behavioral and physiological expressions of pain (Yaksh 2002) as a mechanism to evaluate new and novel pathways and therapeutics. In models of muscular pain, analgesics restore impaired motor

function, indicating that part of the reduction in motor function is associated with sensory hypersensitivity rather than muscle dysfunction. I extended these observations to evaluate motor performance under conditions of neuropathic pain and the impact of agmatine on motor performance in neuropathic mice. To this end, I used the rotating rod (rotarod) assessment commonly used to evaluate motor impairment following drug administration.

Materials & Methods

Animals

Male ICR-CD1 mice (21-24G, Harlan, Madison, Wisconsin) were housed 4 to a cage in a 12-hour light/dark cycle. They were given free access to food and water in a temperature and humidity controlled environment. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Chemicals and Reagents

Agmatine sulfate, dizocilpine maleate ((+)- MK-801), ifenprodil, and putrescine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in 0.9% saline. Anti-agmatine antiserum was generated in guinea pigs as previously described (Wade et al. 2009).

Intrathecal Injections

All drugs were dissolved in sterile saline and delivered in 5-µl volumes via intrathecal injection in conscious mice (Hylden and Wilcox 1980). Briefly, the mice were held by the iliac crest and a 30-gauge, 0.5-inch needle attached to a 50-μL Luer-hub Hamilton syringe delivered 5 μL of injectate into the intrathecal space of the mice.

Spared Nerve Injury

Tactile hypersensitivity was induced using the spared nerve injury model described by Decosterd and Woolf (Decosterd, Allchorne and Woolf 2002). Subjects were placed under isoflurane anesthesia and the left sciatic nerve was exposed, along with its three terminal branches. The common peroneal and tibial nerves were ligated with 5.0 silk suture. The nerves were sectioned 2 mm distal to the ligation site. The sural nerve remained uninjured.

Tactile Hypersensitivity

Mice were placed on a wire mesh grid under a glass enclosure and allowed to acclimate for 30 minutes prior to testing. Hypersensitivity was tested by using an electronic von Frey device (Life Sciences, IITC). The left and right hindpaws were stimulated by the tip of the stimulator with enough force to cause the mouse to withdraw its paw. The amount of force required for withdrawal was recorded in grams. Baseline responses before SNI were collected, and the

%MPE was calculated by the following formula: (Experimental Value-Control)/(Cutoff-Control) X 100. For the purpose of these experiments, the following measurements were used: (Post-Drug Threshold – Pre-Drug Threshold)/(Pre-Surgery Baseline Threshold – Pre-Drug Threshold) X 100.

Motor Coordination

Motor coordination was assessed via an accelerating rotarod (Ugo Basile, Carese, Italy). After a training session, mice were given the opportunity to walk on an accelerating (4-40 rpm) rod for a maximum of 300 seconds. We recorded and compared the latency to fall off of the rotarod between treatment groups.

Statistical Analysis

All statistical analysis was considered significant at $\alpha \leq 0.05$. Mechanical paw withdrawal thresholds collected by von Frey filament stimulation were analyzed by repeated measures ANOVA with Bonferroni post-hoc corrected analysis. Motor coordination data were analyzed by one-way ANOVA.

Results

Agmatine Inhibits Injury-Induced Neuroplasticity

The first goal of this study was to determine agmatine's efficacy at reversing neuropathic pain behaviors as measured by von Frey threshold. To address this question, we utilized the SNI model, a well-characterized model of

neuropathic pain in rodents (Decosterd and Woolf, 2001). Immediately prior to surgery, each mouse was injected intrathecally with a 5 microliter solution of 10 nmol agmatine, as well as on alternating days following surgery (day 2, 4, and 6). Subjects were assessed for their von Frey thresholds prior to surgery and on alternating days after surgery (days 1, 3, 5, and 7) on both the injured (ipsilateral) and non-injured (contralateral) hindpaws. Additional von Frey testing continued weekly for a maximum of 30 days following injury. Mice that received intrathecal agmatine experienced significant attenuation of the development of neuropathic pain past the cessation of intrathecal agmatine injection. These data are represented in Figure 1A.

Additional cohorts were generated and tested in the same manner as the agmatine cohort, but with different pharmacological compounds. Dizocilpine (MK-801), ifenprodil, and putrescine were all intrathecally delivered immediately prior to surgery as well as on days 2, 4 and 6 after injury. Behavioral testing was conducted on days 1, 3, 5, and 7, as well as weekly until a maximum of 30 days following injury. Intrathecal MK-801 inhibited the development of hypersensitivity following SNI. Intrathecal ifenprodil showed reduced efficacy as compared to agmatine and MK-801, and putrescine was ineffective, displaying similar hypersensitivity to the saline controls. These data are represented in Figure 1B-D.

Agmatine Reverses Established Neuropathic Hypersensitivity

We sought to test the hypothesis that agmatine reverses already established hypersensitivity in the SNI model of neuropathic pain. Mice were tested for their von Frey sensitivity thresholds prior to and following SNI. Two weeks following injury, three groups of equal responding were selected and given saline control, 1 nmol agmatine or 10 nmol agmatine intrathecally for 4 injections on alternating days. Behavioral testing was conducted every day following drug injection and up to 30 days following injury. Intrathecal agmatine attenuated the expression of established neuropathic pain, but this attenuation did not persist following the cessation of injection as it did in Figure 1. These data are represented in Figure 2.

Morphine Reverses Neuropathic Hypersensitivity in a Dose-Dependent Manner

Opioid therapy remains the gold standard in treating clinically presented pain (Cooper et al. 2017b, Dosenovic et al. 2017). As such, we sought to confirm and compare the efficacy of centrally delivered morphine at various doses (1, 3, and 10 nmol) in both the early (induction) and late (maintenance) phases of the establishment of chronic neuropathic pain. To test for morphine's early efficacy, animals were assessed for their uninjured von Frey thresholds and then given SNI. On day 3 following injury, mice were assessed for their post-injury von Frey

thresholds on both the injured (ipsilateral) and non-injured (contralateral) hindpaws. 1 nmol morphine was intrathecally injected, followed by additional von Frey assessments at 30, 60, 90, 150, and 180 minutes post-injection. This paradigm was repeated on day 4 (3nmol) and day 5 (10nmol). These data are represented in Figure 3. To assess for morphine's efficacy in the maintenance phase of neuropathic pain, a second cohort of mice were assessed on days 14 (1 nmol), 15 (3 nmol) and 16 (10nmol) post-injury. Morphine displayed a dose-dependent reversal of hypersensitivity following spared nerve injury in both induction and maintenance phases of neuropathic pain. These data are presented in Figure 3.

Agmatine Reverses Neuropathic Hypersensitivity in a Dose-Dependent Manner

We sought to confirm and compare the efficacy of systemically delivered agmatine at various doses (10, 30, 100 mg/kg) in both the early (induction) and late (maintenance) phases of the establishment of chronic neuropathic pain. To test for agmatine's early efficacy, animals were assessed for their uninjured von Frey thresholds and then given SNI. On day 3 following injury, mice were assessed for their post-injury von Frey thresholds on both the injured (ipsilateral) and non-injured (contralateral) hindpaws. 10 mg/kg agmatine was intravenously injected, followed by additional von Frey assessments at 30, 60, 90, 150, and 180 minutes post-injection. This paradigm was repeated on day 4 (30 mg/kg)

and day 5 (100 mg/kg), and these data are represented in Figure 4. To assess for agmatine's efficacy in the maintenance phase of neuropathic pain, a second cohort of mice was assessed on days 14 (10 mg/kg), 15 (30 mg/kg) and 16 (100 mg/kg) post-injury. Agmatine displayed a dose-dependent reversal of hypersensitivity following spared nerve injury in both induction and maintenance phases of neuropathic pain.

Agmatine Attenuates Non-Reflexive Pain Behaviors as Measured by Motor Performance

In addition to reflexive (von Frey) behavioral testing, we sought to assess whether central delivery of agmatine attenuates non-reflexive expressions of neuropathic pain. To this end, we performed SNI on male ICR mice. Prior to injury and following injury, mice were assessed for their motor performance on the rotarod assay. These mice were then separated into equal groups, and injected with 10 nmol agmatine, 10 nmol MK-801, or saline control. 15 minutes following injection, mice were placed on an accelerating rotarod and their latency to fall off of the rotarod was recorded, as presented in Figure 5. SNI inhibited performance in the rotarod assay, as demonstrated by the significant decrease in time spent on the rotarod following injury. Mice injected intrathecally with saline displayed this same impairment of rotarod performance. However, mice injected with 10 nmol agmatine showed a significant increase in time spent on the rotarod, likely due to the analgesia provided by agmatine treatment. Intrathecal

MK-801 significantly inhibited rotarod performance as compared to saline control; this motor impairment is characteristic of widespread NMDA inhibition.

Table 1: Experimental Design for Central Delivery of Agents to Modify Neuronal Injury-Induced Plasticity

Day -1	**Day 0**	**Day 1**	**Day 2**	**Day 3**
von Frey (vF) threshold baseline	i.t. injection of 10nmol study drug or saline Spared nerve injury	vF Threshold	i.t. 10 nmol study drug	vF Threshold
Day 4	**Day 5**	**Day 6**	**Day 7**	**Days 8-30**
i.t. 10 nmol study drug	vF Threshold	i.t. 10 nmol study drug	vF Threshold	vF Threshold

Figure 1

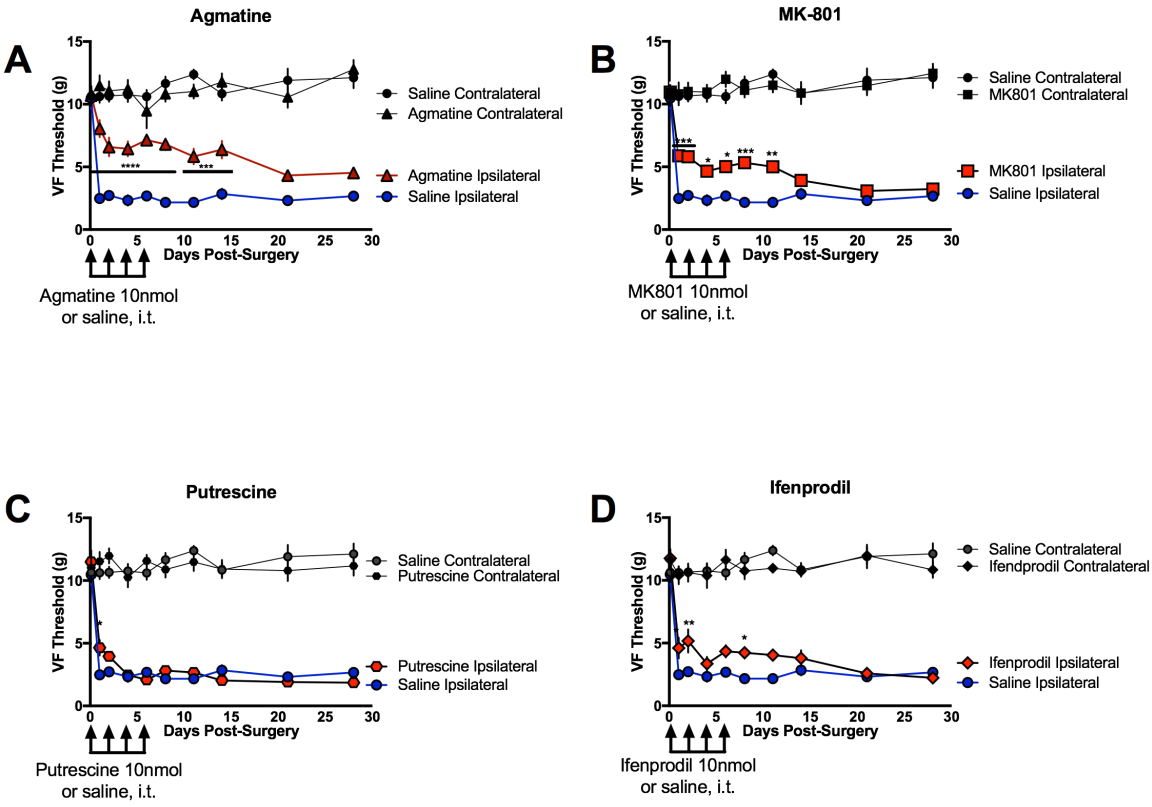

Figure 1: Centrally delivered agmatine attenuates the development of neuropathic pain behaviors following SNI. Behavioral hypersensitivity was measured prior to and following SNI in saline controls (circles) compared to (A) 10 nmol agmatine (triangles), (B) 10 nmol MK-801 (squares) (C) 10 nmol putrescine (hexagons), or (D) 10 nmol ifenprodil (diamonds). All drugs were delivered intrathecally immediately prior to and on days 2, 4, and 6 following spared nerve injury. Data are expressed in grams of force required to elicit a behavioral response. * represents significant difference from saline control, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ANOVA with Bonferroni post-hoc analysis.

Table 2: Experimental Design for Agmatine's Reversal of Hypersensitivity Induced by SNI

Day -1	**Day 0**	**Day 8**	**Day 14**	**Day 15**	**Day 16**
von Frey (vF) threshold baseline	Spared nerve injury (SNI)	vF Threshold	vF Threshold	i.t. 1 or 10 nmol agmatine	vF Threshold
Day 17	**Day 18**	**Day 19**	**Day 20**	**Day 21**	**Days 22-31**
i.t. 1 or 10 nmol agmatine	vF Threshold	i.t. 1 or 10 nmol agmatine	vF Threshold	i.t. 1 or 10 nmol agmatine	vF Threshold

Figure 2

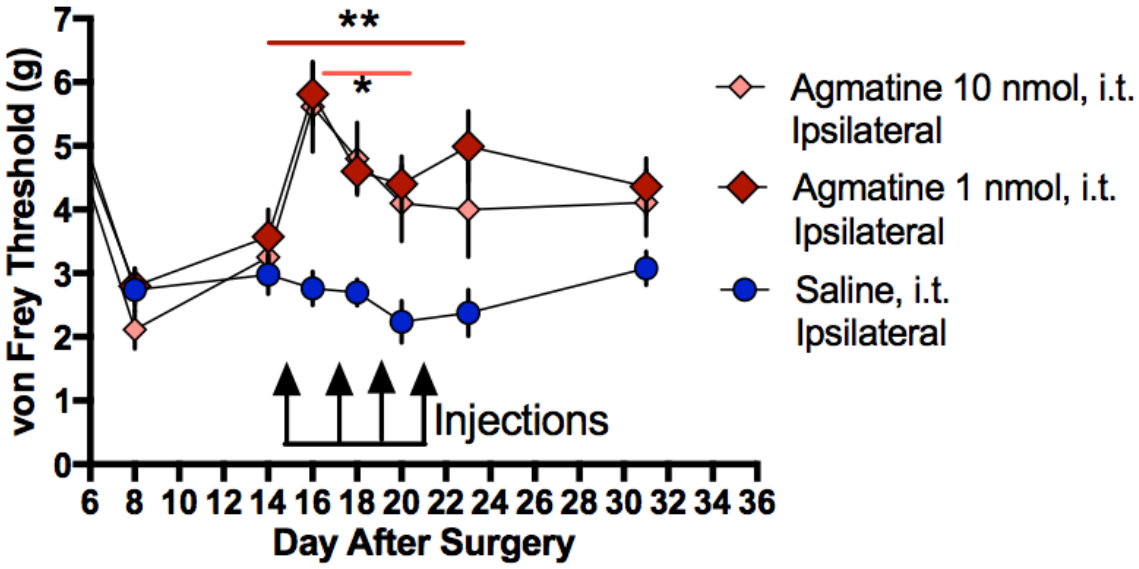

Figure 2: Central delivery of agmatine reverses the expression of hypersensitivity following SNI. All subjects were given SNI to induce local hypersensitivity and split into 3 groups of equal behavioral hypersensitivity. 15 days following injury, subjects were given saline control (circles), 1 nmol agmatine (large diamonds), or 10 nmol agmatine (small diamonds), all delivered intrathecally on alternating days. * represents significant difference from saline control. $p < 0.05$, ** $p < 0.01$, ANOVA with Bonferroni post-hoc analysis.

Table 3: Experimental Design for Morphine's Efficacy at Inhibiting Early and Late Stage Hypersensitivity Following SNI

Induction				
Day -1	**Day 0**	**Day 3**	**Day 4**	**Day 5**
von Frey (vF) baseline	SNI	1 nmol morphine sulfate, intrathecal (i.t.) vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	3 nmol morphine sulfate, i.t. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	10 nmol morphine sulfate, i.t. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection
Maintenance				
Day -1	**Day 0**	**Day 14**	**Day 15**	**Day 16**
vF baseline	SNI	1 nmol morphine sulfate, i.t. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	3 nmol morphine sulfate, i.t. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	10 nmol morphine sulfate, i.t. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection

Figure 3

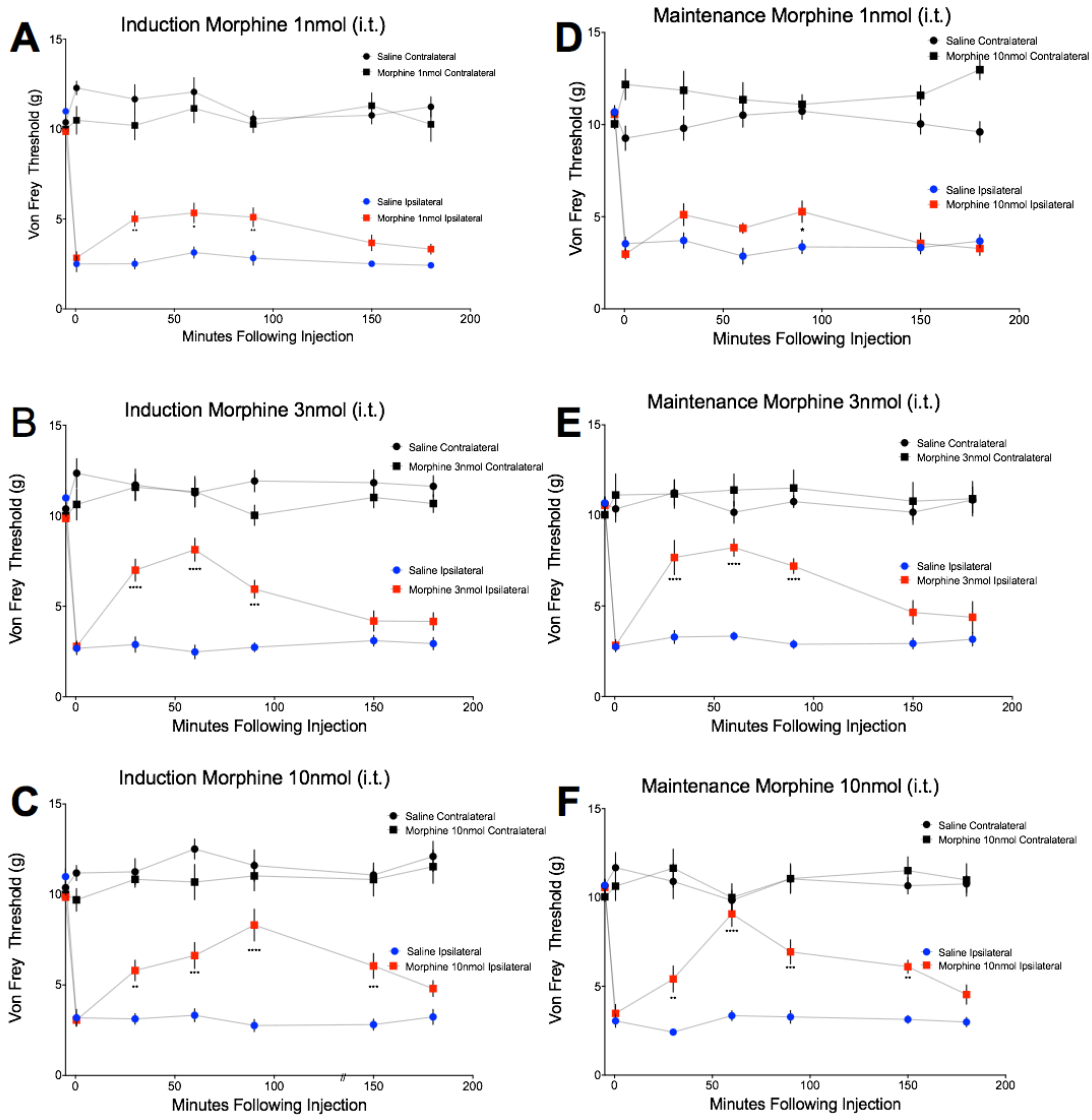

Figure 3: Centrally delivered morphine reverses neuropathic pain behaviors. Behavioral hypersensitivity was measured prior to and following SNI in saline controls (circles) compared to intrathecal morphine (squares) in induction (A-C) and maintenance (D-F) phases of neuropathic pain. Data are expressed in grams of force required to elicit a behavioral response. * represents significant difference from saline control, $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ANOVA with Bonferroni post-hoc analysis.

Table 4: Experimental Design for Agmatine's Efficacy at Inhibiting Early and Late Stage Hypersensitivity Following SNI

Induction				
Day -1	**Day 0**	**Day 3**	**Day 4**	**Day 5**
von Frey (vF) baseline	SNI	10 mg/kg agmatine, intravenous (i.v.) vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	30 mg/kg agmatine, i.v. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	100 mg/kg agmatine, i.v. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection
Maintenance				
Day -1	**Day 0**	**Day 14**	**Day 15**	**Day 16**
vF baseline	SNI	10 mg/kg agmatine, i.v. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	30 mg/kg agmatine, i.v. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection	100 mg/kg agmatine, i.v. vF testing at 0, 30, 60, 90, 150, 180 minutes following injection

Figure 4

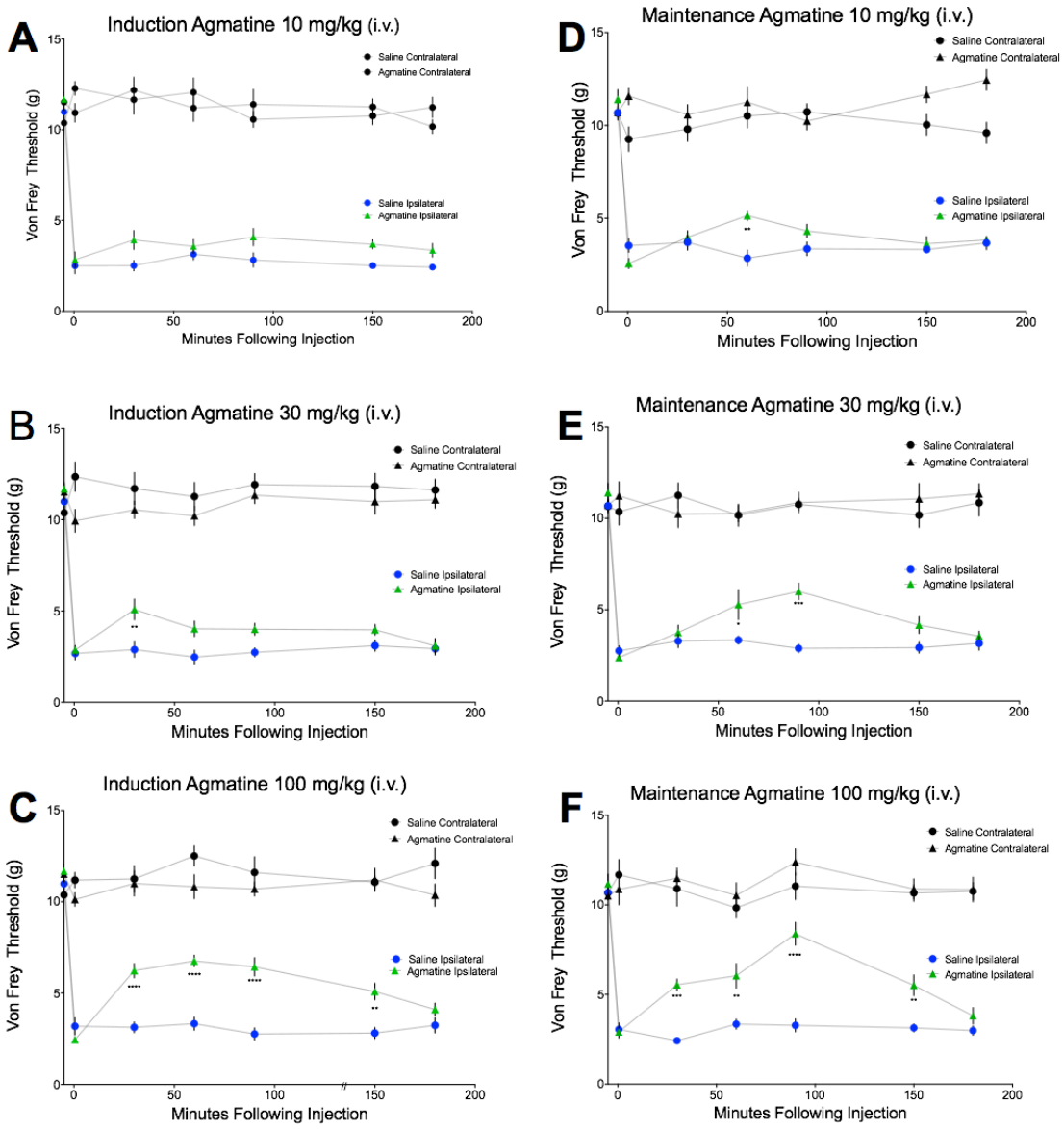

Figure 4: Systemically delivered agmatine reverses neuropathic pain behaviors. Behavioral hypersensitivity was measured prior to and following SNI in saline controls (circles) compared to intravenous agmatine (triangles) in induction (A-C) and maintenance (D-F) phases of neuropathic pain. Data are expressed in grams of force required to elicit a behavioral response. * represents significant difference from saline control, $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ANOVA with Bonferroni post-hoc analysis.

Figure 5

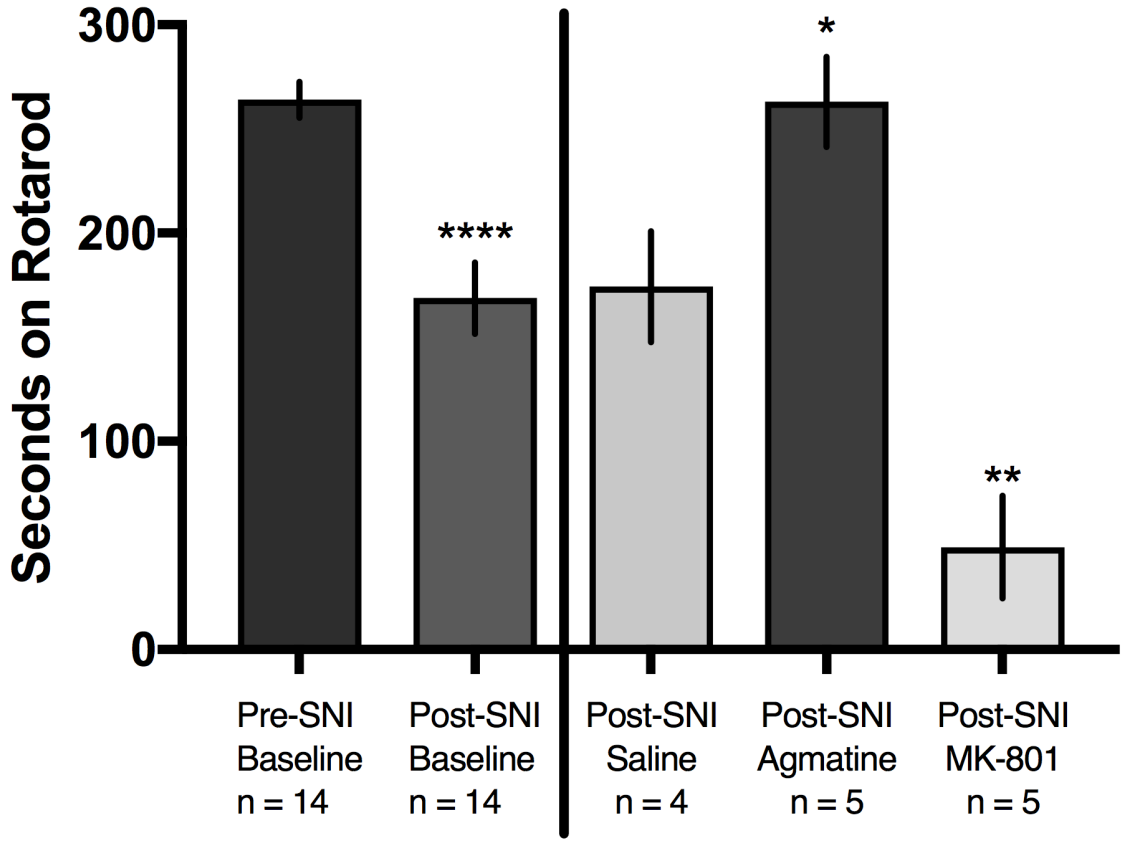

Figure 5: Agmatine attenuates non-reflexive pain behaviors as measured by rotarod performance. All mice were assessed for their baseline motor coordination by rotarod performance with a cutoff time of 300 seconds, then given SNI to induce local hypersensitivity and assessed for their decrement in performance. Students t test, **** $p < 0.0001$. Following injury, subjects were injected with saline, 10 nmol agmatine, or 10 nmol MK-80, i.t and placed on an accelerating rotarod 30 minutes following injection. Latency to fall was recorded, and data were analyzed by one-way ANOVA with reference to the saline control, * $p < 0.05$, ** $p < 0.01$.

Discussion

A key observation in these findings is that central administration of agmatine results in the prevention or reversal of chronic neuropathic pain behavior. Most notably, Figure 1 demonstrates the reduction in chronic pain behaviors for days to weeks following the cessation of injection. Agmatine was delivered a minimum of 12 hours prior to sensory testing, making it likely that the impact of agmatine is one of inhibition of neuronal remodeling instead of acute inhibition of evoked hypersensitivity. The decision to administer intrathecal agmatine every other day and to assess sensory thresholds on the alternate days is in part due to agmatine's 12 hour half-life following central delivery (Roberts et al. 2005).

In addition, we sought to determine if agmatine delivery was sufficient to inhibit or attenuate the neuroplasticity involved in development of neuropathic pain. It is important to note that agmatine attenuated the development of chronic neuropathic pain, in contrast to its metabolite, putrescine. This indicates a direct effect of agmatine rather than a subsequent increase in putrescine following intravenous agmatine delivery as being responsible for the inhibition of maladaptive plasticity following SNI.

Due to NMDA inhibition's long history as a pharmacological target, the side effect profile of a classic NMDA antagonist is well characterized (Olney 1994, Neznanova et al. 2000, Lipton 2004). Motor impairment, psychotic symptoms, and memory impairment are seen in both animal modeling and

clinically available NMDA antagonist therapeutics (Olney 1994, Lipton 2004). In response to these established concerns, we have conducted a battery of side effect monitoring. Presented in Figure 5, we conducted the most widely used assay for motor coordination, rotarod performance, and saw a significant increase as compared to saline controls in the time that neuropathic pain animals intrathecally injected with agmatine were able to walk and balance on the rotarod. In contrast to agmatine's increase, intrathecally delivered MK-801 significantly decreased each subject's motor coordination, as indicated by a decrease in the amount of time they were able to walk on the rotarod.

In addition to time spent on the rotarod, a notable behavioral phenotype was observed where animals dropped their injured paw off of the rotarod and used only their three non-injured paws, presumably due pain-related sparing of the injured paw. This observation aligns with recent publications seeking to characterize non-reflexive measures of pain in animal models, including voluntary wheel running (Grace et al. 2014) grid-climbing (Falk, Gallego-Pedersen and Petersen 2017), voluntary movement such as rearing or distance traveled (Cho et al. 2013), exploratory behavior (Zhu et al. 2012), and dynamic weight bearing (Laux-Biehlmann et al. 2016). Intrathecal delivery of agmatine, shown to be analgesic in evaluations of mechanical hypersensitivity as measured by von Frey stimulation, significantly increased rather than decreased the time that neuropathic animals were able to sustain their position on the rotarod. It is highly probable that agmatine reduced the hypersensitivity of the paw at a resting state,

leading to less pain-related sparing of the injured paw and an increase in the injured paw's use on the rotarod, thus increasing the time spent on the rotarod as compared to the saline injected neuropathic controls. In contrast, intrathecal delivery of MK-801 resulted in motor dysfunction characteristic of wide-spread NMDAR antagonism.

Summary and Conclusion

This study features the safety and efficacy of exogenous central delivery of agmatine as a therapeutic strategy for management of chronic neuropathic pain. The pharmacology of chronically delivered agmatine reported in Figure 1 is comparable and improved in magnitude relative to the gold standard NMDA receptor antagonist MK-801 and the gold standard GluN2B-specific antagonist of the NMDA receptor, ifenprodil, and distinct from that of its metabolite, putrescine, which was inactive. This outcome supports the proposal that agmatine acts as an NMDA receptor antagonist to reduce tactile hypersensitivity arising from nerve injury. The pharmacology of acutely delivered systemic agmatine is comparable to that of morphine in that magnitude of effect, time to onset, and duration of action are comparable, although the potency lower. These data indicate that both systemic and centrally delivered agmatine are effective for providing relief from tactile hypersensitivity with distinct pharmacological profiles. These data contribute to the foundation for translation of safe and effective NMDA inhibition to a clinical setting.

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Chapter 3: Long-Term Reversal of Chronic Pain Through Elevation of Spinal Agmatine

Synopsis

Inhibition of pathological neuroplasticity by exogenous delivery of agmatine has been demonstrated in chronic neuropathic pain, as presented in Chapter 2 of this thesis. The mammalian enzyme arginine decarboxylase (ADC) is thought to synthesize agmatine by decarboxylation of L-arginine. Therefore, we hypothesized that a gene therapy overexpressing ADC may counter the effects of the central nervous system (CNS) disorders mentioned above. In this chapter, I report that the overexpression of the ADC enzyme in tissues relevant to the pain pathway both prevents and reverses neuropathic pain in rodents.

(C.P planned and conducted the experiments and behavioral analysis. K.K. and R.S.P conducted experiments. C.A.F and G.W. contributed to analysis and editing.)

Introduction

Controlling chronic pain remains a significant scientific and clinical challenge and a critical public health concern (IOM 2011). Identification of new pathways and approaches to counter pain arising from maladaptive neuroplasticity is greatly needed. It is well established that chronic pain is generated and maintained due to NMDA receptor-mediated maladaptive neuroplasticity localized in part at the level of the spinal cord, which is the location of the first synapse in the sensory relay. Pharmacological targeting of the NMDA receptor (Yamamoto and Yaksh 1991, Mao et al. 1992, Geng et al. 2010, Chaplan, Malmberg and Yaksh 1997, Decosterd, Allchorne and Woolf 2004, Qu et al. 2009, Fairbanks et al. 2000a) and associated nitric oxide synthase (NOS) cascade has been a broadly pursued strategy for several decades (Yamamoto and Shimoyama 1995, Yoon, Sung and Chung 1998, Guan et al. 2007, Tanabe et al. 2009, Chacur et al. 2010). However, development of this strategy has been limited due to various undesirable side effects related to the action of NMDA receptor antagonists on CNS regions associated with motor and cognitive function.

We and others have previously shown that exogenous administration of the NMDA receptor/NOS inhibitor decarboxylated arginine (agmatine) reduces manifestations of neuronal plasticity in a wide spectrum of models of CNS disorders (Piletz et al. 2013), including chronic pain (Courteix et al. 2007, Fairbanks et al. 2000b, Horvath et al. 1999). Agmatine is distinguished from

most other NMDA receptor antagonists/NOS inhibitors in that it is endogenously produced (Satriano 2004). Additionally, in contrast to the synthetic NMDA receptor antagonists, the effect of exogenous agmatine on CNS side effects appears to be limited or undetected in pre-clinical models of motor dysfunction (Fairbanks et al. 2000b, Nguyen et al. 2003).

We hypothesized that endogenously produced agmatine can modify chronic pain. Agmatine has been proposed to be generated from the enzyme arginine decarboxylase (ADC) (Morrissey et al. 1995), a protein expressed in a variety of tissues including the CNS (Regunathan and Reis 2000). Therefore, it stands to reason that genetic modification of the agmatinergic system to enhance endogenous production of agmatine may reduce consequences of maladaptive neuroplasticity. We have previously demonstrated (Vulchanova et al. 2010) that intrathecal delivery of AAV5-GFP by direct lumbar puncture results in robust gene transfer to the dorsal root ganglia and the spinal cord, particularly at the lumbrosacral and cervical levels. Therefore, we have developed an adeno-associated viral (AAV) vector that carries the gene for ADC to determine whether overexpression of agmatine in tissues relevant to pain transmission can attenuate neuropathic pain behavior in mice. Here we report our design and biological evaluation of the impact of intrathecal delivery of AAV5-hADC vectors that contain the synthetic human gene for agmatine. The observations from this study indicate that overexpression of ADC in dorsal root ganglia and spinal cord

(as well as some supraspinal structures) reduces both the development and maintenance of chronic pain behaviors in nerve-injured rodents.

Materials and Methods

Gene Construct Development

Human ADC cDNA was cloned into the EcoRI and Xho1 sites of plasmid pAAV-IRES-hrGFP (Agilent Technologies). The resulting plasmid pAAV-ADC contains the ADC expression cassette (CMV promoter-ADC cDNA-IRES-GFP and bovine growth hormone polyadenylation signal) flanked by AAV2 inverted terminal repeats (Figure 2A). This vector was packaged into AAV5 and AAV9 virions at the University of Florida Vector Core by co-transfection of HEK293 cells and purified from cell lysates on an iodixanol step gradient followed by Q Sepharose ion exchange chromatography (Zolotukhin et al. 2002). Vector titers were 8.24 or 9.77 x 10^{13} vector genomes/mL for AAV5-ADC-GFP (two separate lots were used) and 6.37 x 10^{13} vector genomes/mL for AAV9-ADC-GFP.

Animals

All experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee. Experimental subjects were Institute of Cancer Research (ICR) male mice (21-30 grams), or Sprague Dawley rats (175-400 grams) both from Harlan (Madison, WI). Subjects were housed in groups of

4 mice or 2 rats per cage in a temperature- and humidity-controlled environment and maintained on a 12 hour light/dark cycle with free access to food and water.

Injectates

Agmatine, naloxone, idazoxan, putrescine, ifenprodil, and MK-801 were purchased from Sigma Chemical (St. Louis, MO) and diluted in 0.9% NaCl. Anti-agmatine guinea pig IgG or normal guinea pig IgG were developed in-house as previously described (Wade et al. 2009). These were protein A-purified to the IgG fraction, concentration-matched, and diluted in 0.9% NaCl at 150 ng/5 microliter injection. All viral vectors used in these experiments were packaged and/or purchased from the Vector Core of the University of Florida, Gainesville, Florida.

Intrathecal Injections

All viral vectors, drugs, or IgGs were administered intrathecally (i.t.) by direct lumbar puncture in conscious rodents as described for mice (Hylden and Wilcox 1980) and rats (Mestre et al. 1994). Briefly, the subjects were gently gripped by the iliac crest and a 30-gauge, 0.5 inch needle (mice) or 27 gauge 1.4 inch needle (rats) connected to a 50-μL Luer-hub Hamilton syringe was used to deliver 5 μL of injectate in the intrathecal space. In the case of viral vector injection, a modified needle, catheter, syringe apparatus was used to inject 10 microliters of injectate in order to conserve product. AAV5-GFP or AAV9-GFP

stocks (purchased from University of Florida, Vector Core, Gainesville, Florida) were diluted to titer match the corresponding AAV-hADC titers prior to injection. In the case of the AAV5 vectors, the hyperosmotic agent mannitol (25%, 200 microliters, tail vein) was delivered as an intravenous pre-treatment to enhance distribution of the AAV5 vectors to the CNS following intrathecal delivery (Vulchanova et al. 2010). By contrast, mannitol pre-treatment does not enhance CNS distribution of AAV9 serotype after intrathecal delivery (Schuster et al. 2014) and so it was not used in the experiments using the AAV9 serotype.

RT-PCR

Total RNA was extracted from tissue or cell lines using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to manufacturer's suggestions. Approximately 1.3 micrograms of this RNA was reverse-transcribed. Briefly, the RNA was added to a reverse transcription (RT) master mix (final concentrations: 2.5 U Multiscribe murine leukemia virus reverse transcriptase, 2.5 mM random hexamers, 200 μM of each dNTP, 5 U RNase inhibitor, 5.0 mM dithiothreitol, 1.75 mM $MgCl_2$, 30 mM tris-HCl and 20 mM KCl; pH 8.3) in a volume of 40 μl. cDNA synthesis was performed in a thermal cycler (BioRad Mini) with the following program: 25°C for 5 minutes (primer annealing), 42°C for one hour (primer extension) and 65°C for 5 minutes (inactivation of the reverse transcriptase). 4.5 μl of RT reaction product was added to 10 μl

Lightcycler 480 SYBR green I Master (Roche, Indianapolis, IN) and 15 μM of each forward and reverse primer in a total of 20 μl per reaction. The sequences of the primers used are shown in Table 2. PCR amplification was performed in thermal cycler (Lightcycler 480 II, Roche) using the following program: 1 cycle of 95˚C for 5 minutes (DNA polymerase activation) followed by 50 cycles of 95˚C for 10 seconds (DNA denaturation), 57˚C for 10 seconds (primer annealing) and 72˚C for 10 seconds (primer extension). PCR amplification was followed by built-in melting temperature and cooling programs. Ten μl of PCR product was fractionated through a 1.5% agarose gel, visualized by ethidium bromide staining and imaged using a digital workstation (BioRad).

Spared Nerve Injury

Spared nerve injury (SNI) was induced in rodents according to the method described by Decosterd and Woolf (Decosterd, Allchorne and Woolf 2002). The left sciatic nerve and its three terminal branches were exposed under isoflurane anesthesia. The common peroneal and tibial nerves were ligated with a 5.0 silk suture and sectioned distal to the ligation, removing 2-4 mm of the distal nerve stump.

Mechanical sensory assessment

All subjects were assessed for responsiveness to mechanical stimulation using an electronic von Frey anesthesiometer (IITC Life Sciences, Woodland Hills, USA). Mice were placed in glass enclosures on an elevated mesh screen and permitted to acclimate for 15-30 minutes prior to stimulation. The electronic von Frey probe was gently applied to each hindpaw until a brisk withdrawal response terminated application of pressure (within seconds). The paw withdrawal thresholds were recorded. All measurements were taken by a single experimenter blinded to genetic and/or pharmacological treatment.

Analysis of AAV Tissue

Tissue extract was diluted 1:20 into 200 mM borate buffer (pH 9.3) to neutralize the TCA and transferred 10 μL to a fresh microfuge tube along with 5 μL Internal Standard (1 μM, piperazine-1-carboxamidine hemisulfate, Oakwood Products). Agmatine spike was 10 μL of 1 μM agmatine sulfate. Borate buffer was added to bring total volume to 55 μL. Each reaction then received 5 μL of NBD-F solution (100 mM, acetonitrile, Molecular Probes), and was quickly mixed and centrifuged, and incubated for 30 min at 60 ^{o}C. After incubation, all reactions were centrifuged 20 min, 16,000 × g, 4 ^{o}C to clarify.

Derivatized samples were injected for 5 seconds at 0.5 psi into a fused silica capillary (50 μm i.d., 39 cm) in a commercial capillary electrophoresis system (MDQ, Beckman-Coulter) with LIF detection (488 nm excitation).

Samples were run at 15 kV for 30 min with 34 mM (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) in 200 mM borate buffer (pH 9.3) running buffer at capillary temperature of either 25 ^{o}C or 50 ^{o}C . Before each run, the capillary was rinsed with 1 M NaOH, diH_2O, and running buffer. Data were exported to Cutter 7.0 for peak integration (Shackman, Watson and Kennedy 2004) and normalization by total protein.

Motor Coordination

Motor coordination was assessed via an accelerating rotarod (Ugo Basile, Carese, Italy). After a training session, mice were given the opportunity to walk on an acceleration (4-40 rpm) rotarod for a maximum of 300 seconds. We recorded and compared the latency to fall off of the rotarod between treatment groups.

RESULTS

Expression of Human Arginine Decarboxylase in the Sensory System

We hypothesized that overexpression of the synthetic enzyme for agmatine, arginine decarboxylase (ADC, Figure 1) (Morrissey et al. 1995), in the sensory neurons of the dorsal root ganglia and spinal cord would reduce neuropathic pain. To test this hypothesis, we generated an AAV5 vector expressing human ADC (hADC) under transcriptional regulation by a CMV promoter (Figure 2). Using human ADC rather than rodent ADC for gene transfer enabled us to independently distinguish between the two ADC forms in transduced tissue and, therefore, assess the efficacy of gene transfer to the spinal cord and dorsal root ganglia. In Figure 2B**,** we show that species-specific primers can detect native human hADC present in human embryonic kidney (HEK) 293 cells, but do not detect mouse ADC in spinal cord tissue extracted from mice treated with control AAV5-GFP vector. These primers also fail to detect mouse ADC in mouse fibroblast 3T3 cells, but detect hADC in 3T3 cells treated with AAV5-hADC vector. These experiments confirm the specificity of the primers for human ADC. In two independent experiments, $8.7 \times 10^{(11)}$ total vector genomes (10 microliters) of AAV5-hADC were injected intrathecally by direct lumbar puncture in nerve-injured mice. We evaluated the expression of hADC at lumbar and cervical levels both in spinal cord and DRG. RT-PCR analysis revealed transgene expression along the spinal cord and further rostrally into the brain (Figure 2C).

The tissue distribution is summarized in Table 1.

Intrathecal Pretreatment with AAV-hADC Reduces of Neuropathic Pain Behaviors in Mouse and Rat

Six weeks following intrathecal injection of either AAV-hADC, AAV-GFP (control for vector and GFP effects), and saline (control for injection effects), the sensory responses to mechanical stimuli were evaluated prior to and following unilateral spared nerve injury in mouse (Figure 3A) and rat (Figure 3B). AAV-hADC treatment resulted in an increasing significant elevation of paw withdrawal thresholds in injured subjects over the testing period whereas treatment with AAV-GFP or saline had no effect on paw withdrawal thresholds. These data demonstrate that the additional expression of human arginine decarboxylase in DRG, spinal cord or choroid plexus results in a reduction in neuropathic pain behaviors.

Anti-Agmatine IgG Reverses AAV5-hADC Inhibition of Neuropathic Pain

To further determine whether the reduced neuropathic pain responses in AAV5-hADC treated mice were attributable to an effect of the agmatine molecule, we used an alternative indirect approach: immunoneutralization. We assessed the impact of intrathecal delivery of an immunoneutralizing antibody (IgG-Ag) (Wade et al. 2009) on the antihyperalgesic effect observed in AAV5-hADC-

treated mice. In this experiment, a second larger cohort of mice (n = 24) were treated with AAV5-ADC in order that there would be sufficient numbers of transduced subjects to test with anti-agmatine IgG and to compare with controls injected with normal IgG. An additional set of subjects included a smaller cohort (n = 8) of mice treated with AAV5-GFP to represent the expected level of nerve injury-induced hyperalgesia. On day nine after SNI surgery, the AAV5-hADC treated subjects were divided into two separate groups with equivalent levels of hypersensitivity. Twelve mice received anti-agmatine IgG (150 ng/5 μL) and twelve mice received normal IgG (150 ng/5 μL). Those injected with anti-agmatine IgG, but not normal IgG, showed significantly reduced von Frey thresholds at 30 minutes post-injection (Figure 4), a result repeated a week later with additional time points included: 2 and 4 hours post-injection. To exclude the possibility that other endogenous analgesic substances may contribute to the observed effect, the opioid receptor antagonist naloxone and the alpha-2 adrenergic antagonist idazoxan were tested for reversal of the effects of AAV5-hADC at doses confirmed to be effective against their respective agonists (morphine and clonidine, data not shown). Neither antagonist had an effect on the withdrawal thresholds of AAV5-hADC-treated mice, ruling out potential contribution of opioid neuropeptides or norepinephrine. The reversal of the effects of AAV5-hADC by treatment with the anti-agmatine IgG supports the proposal that the overexpression of hADC generates endogenous agmatine, leading to the observed anti-hyperalgesia in AAV5-hADC-treated subjects.

Treatment of Nerve-Injured Mice with AAV9-hADC Reduces Mechanical Hypersensitivity

Therefore, we evaluated whether hADC gene transfer following nerve injury could similarly alleviate previously established hypersensitivity. Nerve-injured mice with mechanical hypersensitivity were injected with either AAV9-hADC or saline on day 8 post-surgery. As early as day 24 post-injection (day 32 post-injury), the AAV9-hADC-treated subjects showed significantly reduced hyperalgesia compared to saline-injected controls (Figure 5). These data indicate that gene transfer of hADC following an established chronic pain state significantly reduced hypersensitivity within 3 weeks of injection, an effect that persisted during the subsequent five weeks of testing. The AAV9-hADC-treated subjects were then divided into two groups with equivalent sensory thresholds and the observed anti-hyperalgesia was assessed for agmatine dependence using the immunoneutralization approach described previously. Those injected with anti-agmatine IgG, but not normal IgG, showed significantly reduced von Frey thresholds at 4 hours post-injection, suggesting that agmatine contributes to the effect of AAV9-hADC.

Reversal of Long-Established Neuropathic Hypersensitivity by Agmatine Production via AAV-hADC

Since nerve injury and the subsequent development of chronic pain are often not predictable and takes significant time to establish, patients most often present to the clinic following long establishment of their chronic pain. Therefore, in order to more closely model that time frame pre-clinically, we next sought to evaluate whether hADC gene transfer following nerve injury could alleviate long-established hypersensitivity. Mice were evaluated for their baseline von Frey behavioral thresholds, then hypersensitivity was induced with SNI surgery. Mice were evaluated again for von Frey thresholds 256 days following nerve injury, split into groups of equal responding, and injected intrathecally with saline or AAV5-hADC on day 266 following injury (Figure 6). The AAV5-hADC-treated subjects showed significantly increased von Frey thresholds compared to their saline counterparts for the duration of the experiment, up to 95 days post-injection and a year post-injury, at which time both cohorts were sacrificed and tissue was collected for bioanalytical analysis, which confirmed both presence of ADC mRNA in sensory tissues and elevated agmatine in lumbar spinal cord (Fairbanks, unpublished observations). This experiment is important because it demonstrates an ability to alleviate chronic pain of long duration, a form of chronic pain that is known to be particularly difficult to treat and manage.

AAV5-hADC Partially Restores Impaired Motor Performance in Nerve-Injured Mice

In Chapter 2, I reported that intrathecal delivery of agmatine improved rotarod performance of nerve-injured subjects, likely due to a reduction of hypersensitivity in the injured paw. I sought to perform a direct parallel of that experiment using AAV9-hADC gene transfer rather than exogenous agmatine. Four cohorts were run in parallel: naïve control, sham-injured control (a muscular incision was made but the nerves remained undisturbed), nerve-injured subjects that received intrathecal saline, and nerve-injured subjects that received intrathecal AAV9-hADC to increase spinal agmatine. Mice were placed on an accelerating rotarod and their latency to fall off was recorded (Figure 7). There was no difference in performance between naive subjects and subjects that received sham surgery. However, there was a significant decrement in performance between subjects that received sham surgery and subjects that received nerve injury with intrathecal saline treatment, indicating that the hypersensitivity following nerve injury is responsible for this large decrement in performance. Subjects that received intrathecal AAV9-hADC following nerve injury displayed significant improvement over their saline-injected, nerve-injured controls, likely due to agmatine's analgesic effect in the hypersensitive paw.

Figure 1

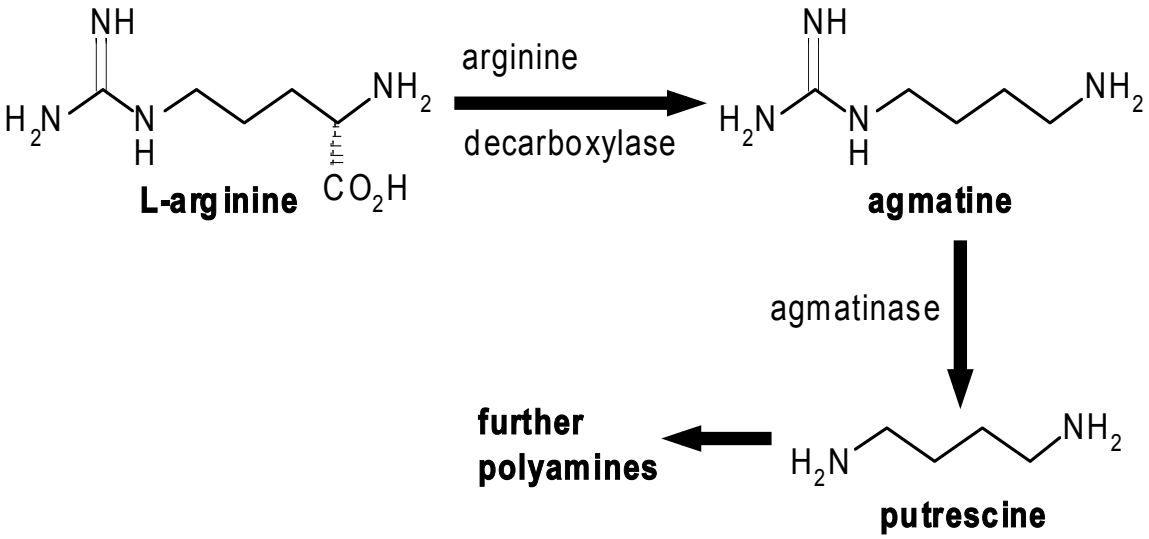

Figure 1: Agmatine synthesis and degradation. Agmatine is synthesized by arginine decarboxylase (ADC) from L-arginine. Agmatine is metabolized by agmatinase (AGM) into putrescine.

Figure 2

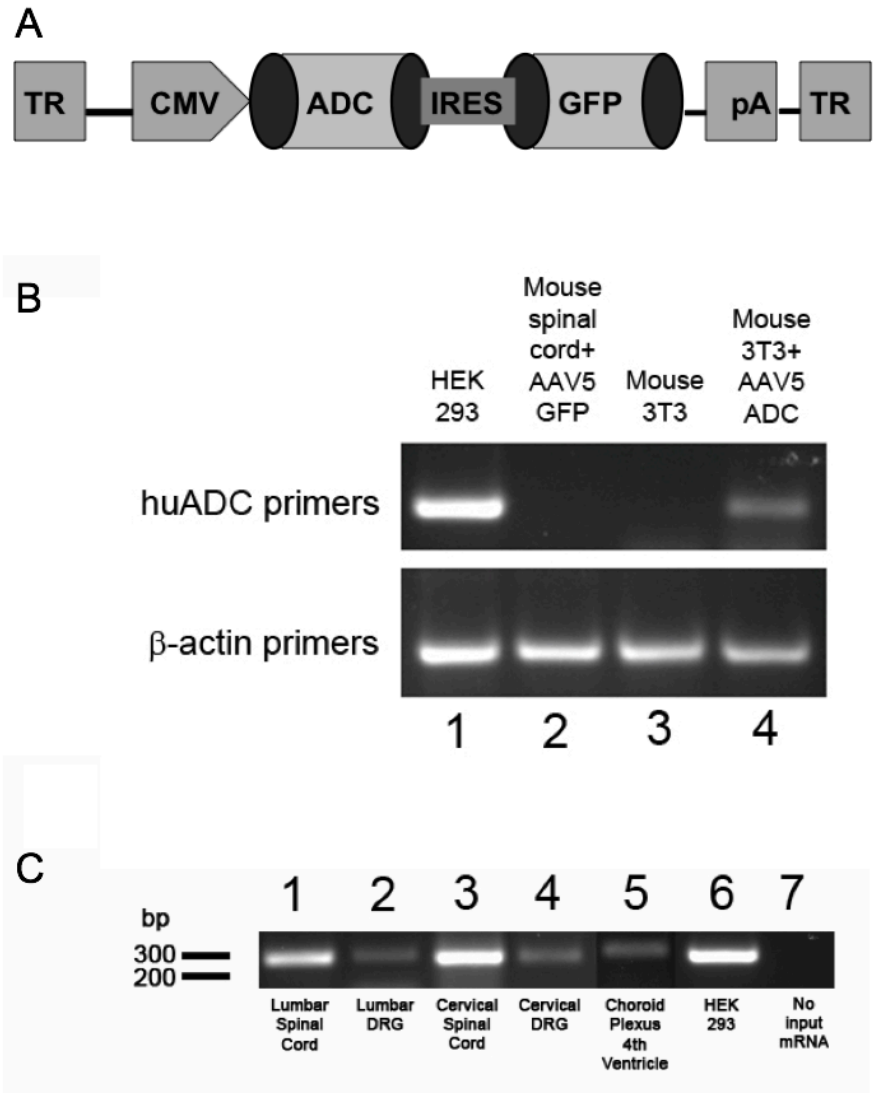

Figure 2: Gene construct for AAV5-hADC and AAV9-hADC viral vectors. A) TR - terminal repeats, CMV – cytomegalovirus, ADC – arginine decarboxylase, IRES – internal ribosome entry site, GFP – green fluorescent protein, pA – poly adenylation. B) Specificity of human ADC as measured in HEK 293 cells, mouse spinal cord injected with AAV5-ADC, mouse 3T3 cells, and mouse 3T3 cells treated with AAV5-hADC. C) Expression of hADC in DRG following AAV

Figure 3

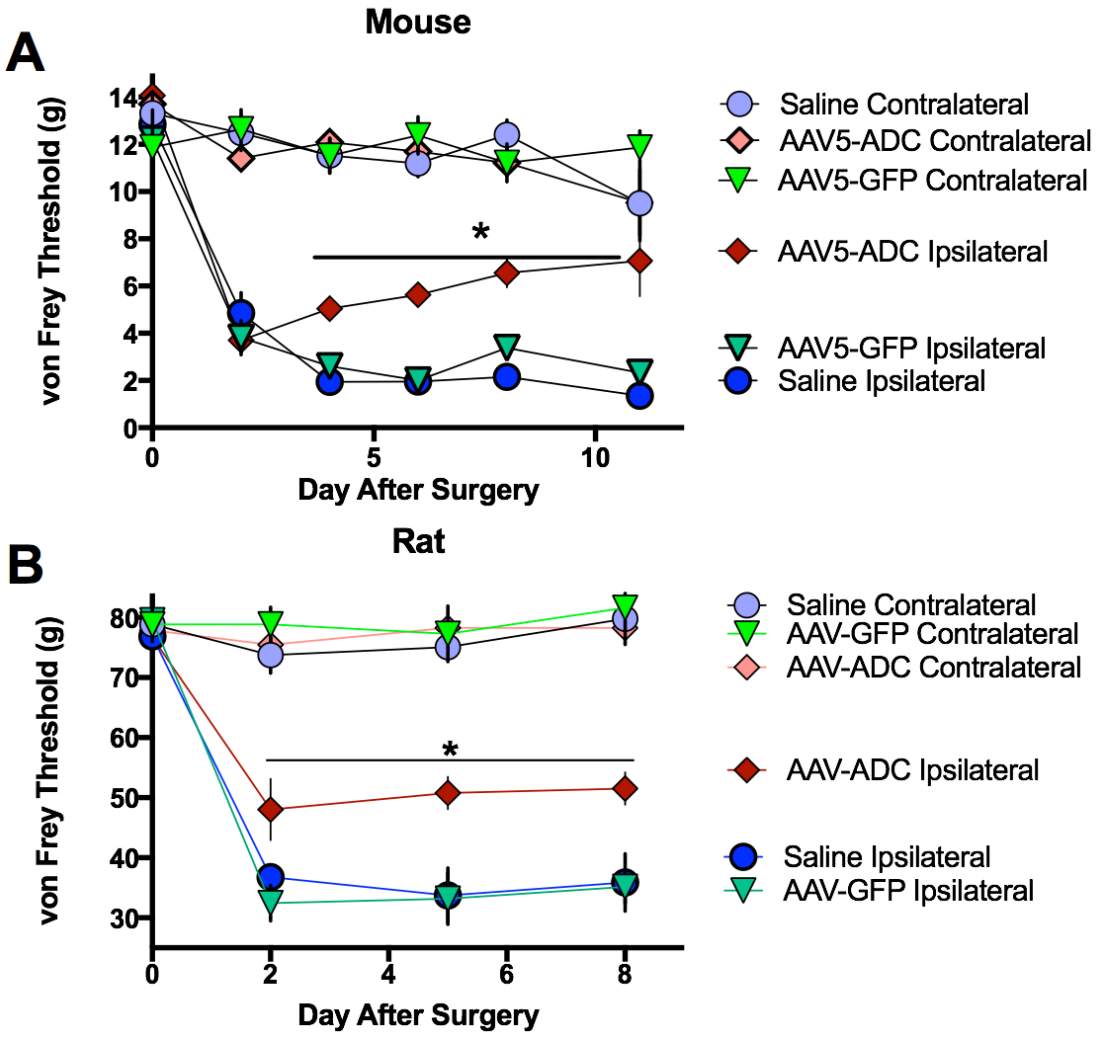

Figure 3: AAV5-hADC pre-treatment inhibits hypersensitivity following neuropathic injury in mice and rats. A minimum of 4 weeks prior to spared nerve injury, mice (A) or rats (B) were intrathecally injected with AAV5-hADC (diamonds), AAV5-GFP (inverted triangles) or saline control (circles). von Frey mechanical testing was performed on injured (ipsilateral) and non-injured (contralateral) paws both prior to and following injury, reported here in grams of force. * represents significant difference from saline control. $p < 0.05$, ANOVA with Bonferroni post-hoc analysis.

Figure 4

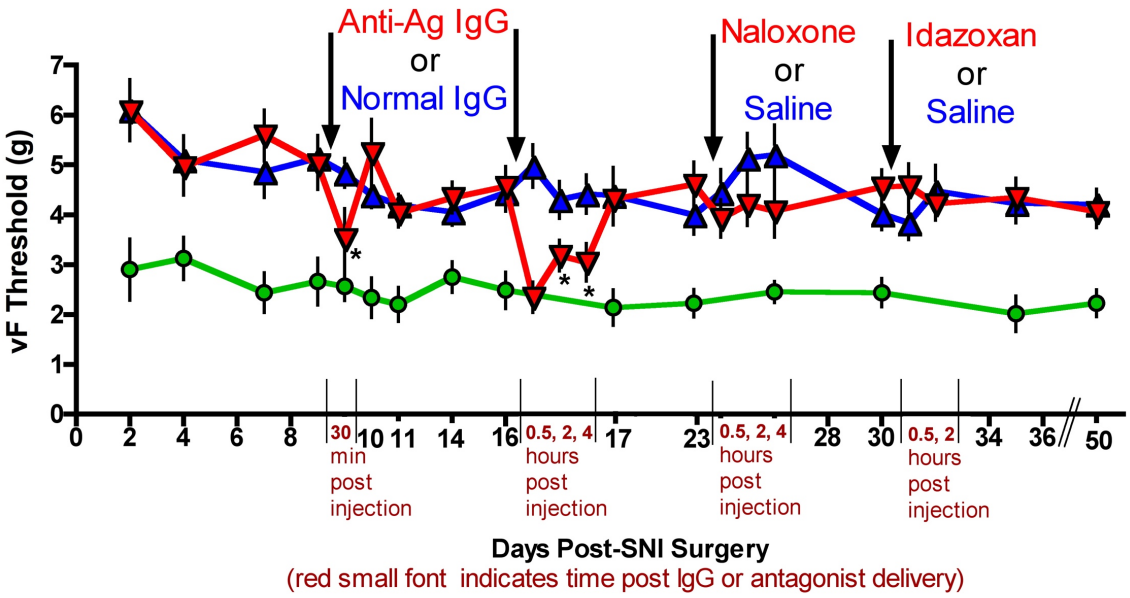

Figure 4: Immunoneutralization of agmatine re-establishes hypersensitivity following AAV-ADC treatment in neuropathic pain. Mice were injected with either AAV5-GFP (control, circles) or AAV9-hADC 6 weeks prior to SNI. Following establishment of injury, AAV9-hADC mice were separated into two groups of equal von Frey thresholds (upright triangles and inverted triangles). On days 9 and 16, these two groups were injected with either normal IgG (upright triangles) or anti-agmatine IgG (inverted triangles) and von Frey thresholds were assessed. On day 23, this paradigm was repeated with naloxone (inverted triangles) or saline (upright triangles). On day 30, this paradigm was repeated with idazoxan (inverted triangles) or saline (upright triangles). * represents significant difference from saline control. $p < 0.05$, ANOVA with Bonferroni post-hoc analysis.

Figure 5

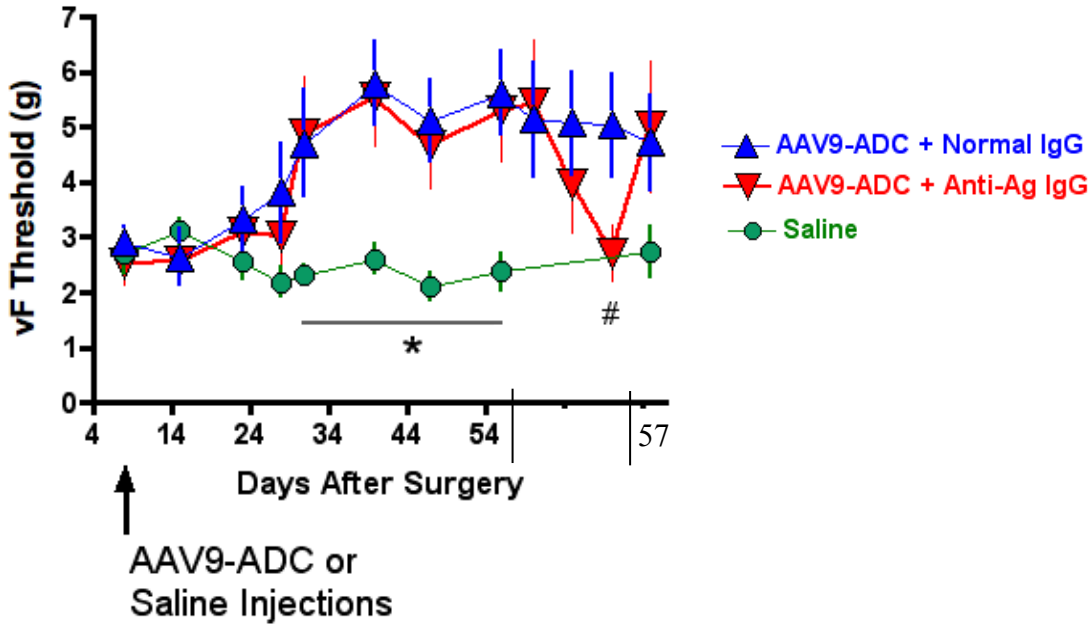

Figure 5: Immunoneutralization of agmatine re-establishes hypersensitivity following AAV-ADC post-treatment of neuropathic pain. One week following spared nerve injury, mice were separated into two groups of equal von Frey mechanical thresholds and intrathecally injected with saline (circles) or AAV9-hADC. On day 54 following injury, mice that received AAV9-hADC were separated into two groups of equal responding; the first group received anti-agmatine IgG (inverted triangles) and the second group received normal IgG (upright triangles). von Frey thresholds were taken 1 and 2 hours following injection. * represents significant difference from saline control. $p < 0.05$, ANOVA with Bonferroni post-hoc analysis. # represents significant difference from normal IgG, $p < 0.05$, Student's t-test.

Figure 6

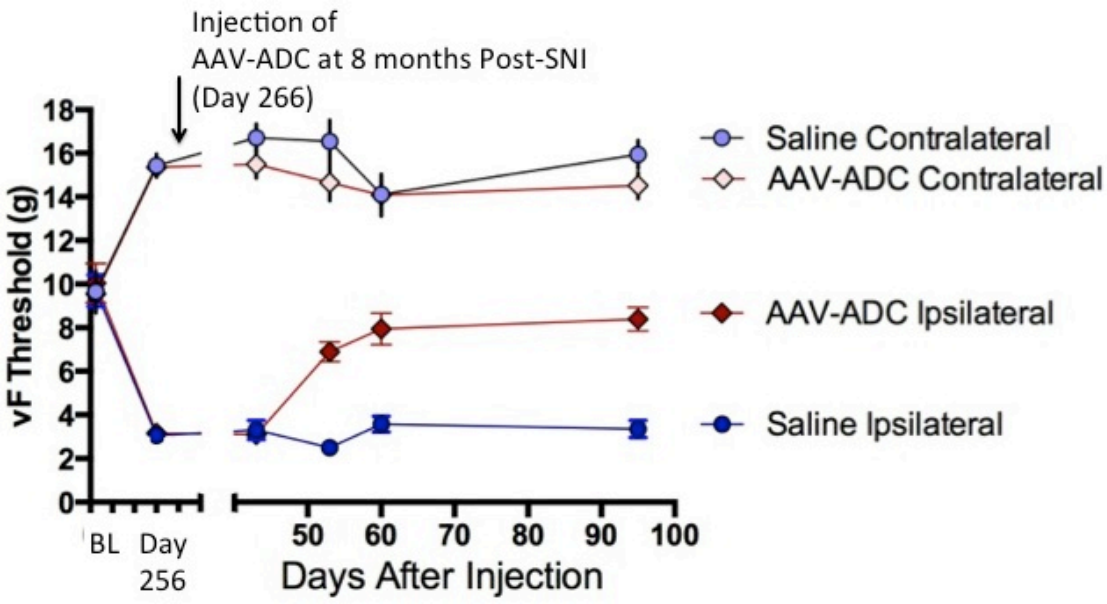

Figure 6: Reversal of long-established neuropathic hypersensitivity by agmatine production via AAV-hADC. All mice were assessed for their baseline mechanical hypersensitivity via von Frey probe and then given spared nerve injury to induce local hypersensitivity. Mice received i.t. saline (circles) or AAV5-hADC (diamonds) 266 days following injury and hypersensitivity was measured up to 95 days following injection. Following completion of behavioral analysis, spinal cord tissue was collected and analyzed for agmatine content.

Figure 7

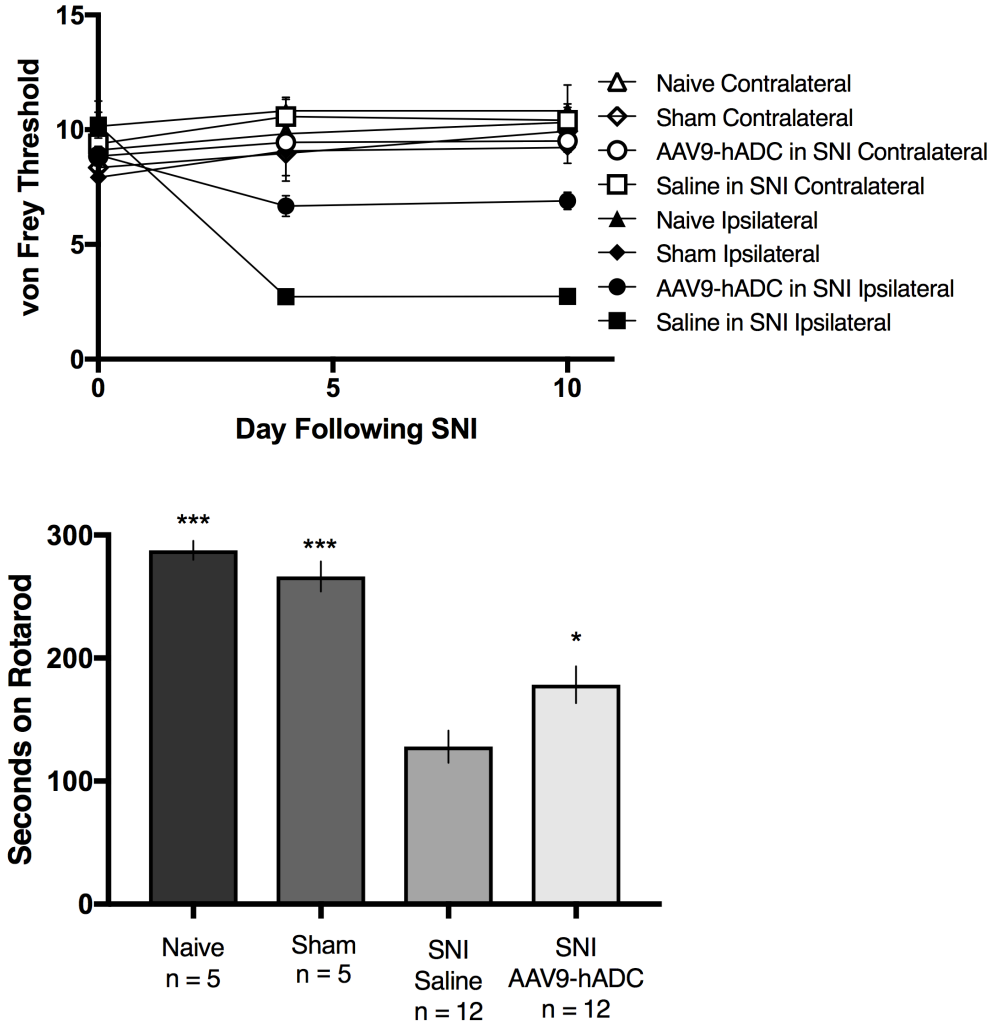

Figure 7: AAV5-hADC partially restores impaired motor performance in nerve-injured mice. Four groups of mice were run in parallel: Non-injured controls (naïve), sham surgery controls lacking neuropathic injury (sham), mice that received saline treatment prior to neuropathic injury (SNI Saline), and mice that received AAV9-hADC prior to neuropathic injury (SNI AAV9-hADC). All mice were assessed for their von Frey thresholds (A) and motor coordination (B) by rotarod performance with a cutoff time of 300 seconds. Latency to fall was recorded, and data were analyzed by one-way ANOVA with reference to the injured saline control, $p < 0.05$ *** $p < 0.001$.

Table 1: mRNA Expression in Tissue Following AAV5-hADC i.t. Injection

Tissue Region Day Post SNI	**Lumbar Spinal Cord**	**Cervical Spinal Cord**	**Lumbar DRG**	**Cervical DRG**	**Choroid Plexus (4^{th} Ventricle)**	**hADC expression in any tissue**
Day 11 (n=19)	*	**61%** (11/18)	**37%** (7/19)	**42%** (8/19)	*	**74%** (14/19)
Day 51 (n=23)	**74%** (17/23)	**95%** (21/22)	**33%** (7/21)	**61%** (13/21)	**57%** (13/23)	**96%** (22/23)

Note: the denominators do not always reflect samples from the entire cohort. In some cases, the samples were not collected or collectable. In some cases, the RNAzol extraction procedure yielded less than 1.3 μg mRNA.
*Tissues not analyzed by PCR

hADC mRNA expression in spinal cord and dorsal root ganglia (DRG) at 53 and 92 days following the initial intrathecal injection of AAV5-hADC. These days corresponded respectively to day 11 and Day 51 post-SNI surgery. Spinal cord (lumbar, cervical), DRG (lumbar, cervical) and choroid plexus (4^{th} ventricle) tissues were microdissected from AAV5-hADC injected mice. The tissues were assayed by RT-PCR for the presence of hADC mRNA as determined by agarose gel electrophoresis.

Table 2. Sequences of Primers Used in RT-PCR Experiments

Name of Primer	Sequence
hADC forward set 2	GCCTTGGACCTGTACTTCCC
hADC reverse set 2	CTGGTCCGTGGATGGTTTCT
b-actin forward qPCR	TCATGTTTGAGACCTTCAACAC
b-actin reverse qPCR	ATGTCACGCACGATTTCCC

Discussion

We and others have previously demonstrated that intrathecally or systemically delivered agmatine reduces multiple manifestations of neuroadaptive pathological plasticity (Piletz et al. 2013), including neuropathic pain as was discussed in Chapter 2 (Courteix et al. 2007, Onal et al. 2003, Karadag et al. 2003, Fairbanks et al. 2000b). The current study illustrated that the pain pathway-related regions of the dorsal root ganglia and spinal cord were transduced with the human form of the enzyme arginine decarboxylase following intrathecal delivery of AAV5 of AAV9 vector carrying the gene for the enzyme. This enzyme is known as the synthetic enzyme of the arginine metabolite, agmatine (Morrissey et al. 1995). Following gene transfer of the human form of arginine decarboxylase, the neuropathic pain behaviors were significantly reduced in magnitude and remained reduced for several months post-injection. A logical explanation is that elevated production of endogenous agmatine could account for such a behavioral response, analogous to what is observed with exogenous delivery of agmatine seen in Chapter 2. A significant finding of these studies is the ability of this gene transfer to reduce long-established hypersensitivity (Figure 6), up to 266 days post-injury. This finding greatly strengthens the viability of translation of agmatine and ADC gene transfer for use in a clinical setting.

In order to test the validity of the above explanation that endogenous

agmatine arising from the AAV-hADC treatments was responsible for the antihyperalgesic effects, we pursued an indirect approach. We applied an immunoneutralization strategy using scavenging antisera to determine whether endogenous agmatine accounted for the anti-hyperalgesic effects of the AAV-hADC treatment. This method has been widely used to assess the effects of endogenous analgesic substances (Vanderah et al. 1994, Tseng et al. 2000, Ohsawa et al. 2001) and recently endogenous pro-nociceptive substances (Fairbanks et al. 2014). We previously demonstrated that a structure-specific anti-agmatine (AG) immunogammaglobulin (IgG) reversed the pharmacological effects of exogenously applied intrathecal agmatine (Wade et al. 2009). We therefore reasoned that intrathecal administration of the anti-agmatine (AG) IgG antibody should similarly reverse the pharmacological effects of endogenous agmatine presumed to be produced by the overexpression of hADC. In fact, when immunoneutralizing antibodies selective for agmatine were delivered intrathecally, the magnitude of the reduction in neuropathic pain behavior was significantly reduced. The selectivity of this effect for agmatine is indirectly supported by the observations that such a reduction was not observed with normal IgG, the opioid receptor antagonist naloxone, or the alpha2 adrenergic antagonist, idazoxan. Specifically, the lack of effect of the normal IgG indicates that anti-Ag IgG reversal of the effect of AAV-hADC overexpression on neuropathic pain is not a random effect of the IgG. Further, the observation that intrathecal naloxone and idazoxan both fail to have an effect indicates that the

effect of AAV-hADC overexpression on neuropathic pain is not due to random compensatory responses of the opioidergic or noradrenergic endogenous analgesic systems. Therefore, these observations support the proposal that elevated endogenous agmatine contributes to the anti-hyperalgesic effect of the AAV5-ADC treatment.

In addition to von Frey mechanical testing, we also probed for a non-evoked measurement of hypersensitivity following nerve injury. To this end, we assessed rotarod performance in naïve, sham-injured, nerve-injured with saline control, and nerve-injured with AAV9-hADC treatment (Figure 7). Similar to my finding in Chapter 2, mice that received AAV9-hADC therapy were able to walk on the accelerating rotarod for a significantly longer amount of time than their saline-treated, injured controls. This outcome likely results from a decrease in sensitivity upon use of the paw to continue walking on the accelerating rotarod.

Taken together, these results indicated that gene therapy, applied to enhance the agmatinergic system, may be feasible for management of chronic pain. When considering that pharmacologically delivered agmatine has other therapeutic effects, it seems likely that other forms of maladaptive neuroplasticity may be suitable candidates for AAV-ADC treatment. However, to date, the agmatinergic system has been minimally investigated; expanded scholarship is greatly needed to understand the relationship of this system to that of pathological and physiological neuroplasticity as well as its general role in the

CNS in order to fully understand and assess its potential for therapeutic applications.

Summary and Conclusion

We have demonstrated that a single intrathecal injection (either before or after the establishment of nerve injury) of AAV vector carrying the gene for arginine decarboxylase results in persistent reduction of chronic pain, in an apparently agmatine-dependent manner even for long-established chronic pain. An emerging literature suggests that such a gene therapy approach is likely to be clinically translatable with a large gene load capacity and long-term expression (Beutler and Reinhardt 2009) which may be valuable for certain chronic pain conditions for which long term pharmacological treatments have challenges. Intrathecal delivery of AAV-ADC will be assessed in further studies to determine the effectiveness in other pain conditions.

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Chapter 4: Agmatine Requires the GluN2B Subunit to Inhibit NMDA Receptor-Mediated Neurobehavioral Plasticity

Synopsis

The data presented in Chapters 2 and 3 strongly support agmatine's action as an antagonist of the NMDA receptor. Based on our preliminary data, we hypothesized that a subunit of the NMDA receptor, specifically GluN2B, is required for agmatine's effect at this receptor. In order to confirm the hypothesis that agmatine requires the 2B subunit of the N-methyl-D-aspartate receptor (NMDAR) to inhibit neuronal signaling, it is necessary to test this using a genetic knock down approach. The study presented in this chapter utilized a GluN2B-floxed mouse line developed at Vanderbilt University to test whether GluN2B-containing NMDA receptors are required for agmatine's reduction of neuropathic pain and opioid analgesic tolerance.

(C.P planned and conducted the experiments and behavioral analysis. K.K. conducted experiments. H.V conducted molecular analysis. C.A.F and G.W. contributed to analysis and editing.)

Introduction

Agmatine, the small molecule synthesized from L-arginine via arginine decarboxylase (ADC), has long been thought to act as an endogenous neurotransmitter/neuromodulator. Agmatine, ADC, and agmatine's degradative enzyme agmatinase are all expressed in mammalian tissues (Li et al. 1994, Raasch et al. 1995). Agmatine was first described as an endogenous agonist of imidazoline receptors (Li et al. 1994). A study examining glutamate-induced neurotoxicity conducted in HEK-293 cells concluded that agmatine likely interacts with a site located within the channel pore of the NMDA receptors expressing NR1-1a and NR2C subunits (Olmos et al. 1999). Further, agmatine has been directly shown to inhibit NMDA receptor channels in whole-cell patch clamp studies, where it produced a concentration- and voltage-dependent block of NMDA receptor currents (Yang and Reis 1999). Despite the foundation of these studies, no functional *in vivo* assay of agmatine efficacy has previously been conducted to probe the necessary composition of the NMDA receptor for the inhibitory effects of agmatine in models of neuropathic pain or opioid tolerance.

NMDA Receptor Characteristics Following Neuropathic Pain

The NMDA receptor is composed of four subunits (Furukawa et al. 2005), typically composed of two GluN1 and two GluN2 subunits (Paoletti, Bellone and Zhou 2013). Of the GluN2 subunits, four subtypes exist (A-D), with each having differential expression and functional properties across the central nervous

system and throughout development (Traynelis et al. 2010), and each encoded by a separate gene (Kutsuwada et al. 1992). The subunit composition determines the pharmacological and physiological characteristics of each NMDA receptor (Monyer et al. 1992); NR1/NR2A receptors display a faster inactivation rate than NR1/NR2B receptors (Vicini et al. 1998).

NMDA receptor activity is altered in several ways following peripheral nerve injury. The NR1 subunit experiences a significantly increased phosphorylation level in dorsal spinal cord and gracile nucleus ipsilateral to the site of injury as compared to the contralateral side (Ultenius et al. 2006, Gao et al. 2005). Wind-up (the ramping up of the number of spikes evoked by repeated C-fiber stimuli arising from the periphery (Herrero, Laird and López-García 2000)) following peripheral injury can be inhibited by NMDA antagonists such as ketamine, MK-801 and the GluN2B-selective antagonists memantine, RO-25 6981, and ifenprodil (Suzuki, Matthews and Dickenson 2001, Qu et al. 2009). Analysis of the GluN2B-selective NMDA antagonists indicates GluN2B-containing NMDA receptors are critical to the development of neuropathic pain at early stages following injury, and for the development of long-lasting enhanced spinal excitability.

Further evidence supporting GluN2B's involvement in the spinal plasticity and central sensitization of pain lies in the tyrosine phosphorylation of this 2B subunit and its increase in multiple pain states (Luo et al. 2014, Liang et al. 2017, Bu et al. 2015, Guo et al. 2002). NMDA receptor responses to agonists are

enhanced following phosphorylation (Zhou, Chen and Pan 2011). Following injection of complete Freund's adjuvant, a prolonged increase in tyrosine phosphorylation of GluN2B but not GLuN2A is correlated to the temporal expression of hyperalgesia and inflammation (Guo et al. 2002). Injection of saline results in only a transient increase in tyrosine phosphorylation of NR2B, indicating that the phosphorylation of GluN2B is maintained by primary afferent input from the site of injury. Taken in total, these data indicate that targeting the GluN2B subunit of the NMDA receptor following neuropathic pain is a viable strategy to reduce neuropathic pain.

Probing the GluN2B Subunit *in vivo*

Pharmacologically, compounds such as ifenprodil (Gallagher et al. 1996), RO 25-6981 (Lynch et al. 2001), and polyamines and protons (Gallagher et al. 1997) demonstrate selectivity for the 2B over the 2A subunits of the NMDA receptor. Probing the physiological function and relevance of the 2B subunit of the NMDA receptor has historically been difficult to resolve. One early study utilizing mutant mice deficient in this subunit concluded that GluN2B was essential for synaptic plasticity and neuronal pattern formation; these mice lacked a suckling response and died shortly after birth unless hand-fed (Kutsuwada et al. 1996), likely due to NMDA-mediated developmental regulation (Hestrin 1992). The hippocampus of these mutant mice did not respond to a standard long-term depression (LTD) protocol and lacked synaptic NMDA responses in the

trigeminal nucleus. These results indicate that the 2B subunit is required for development, synaptic plasticity and neuronal pattern formation such as in the formation of memory.

Advances in gene editing technology led to the development of GluN2B-floxed mice (Brigman et al. 2010). These mice were initially crossed with transgenic mice expressing CAMKII-driven Cre recombinase, which enabled the production of mice with reduced GluN2B expression in neurons of the cortex and CA1 region of the hippocampus. Further studies utilizing these mice have indicated their viability for use in a site-specific knock down of GluN2B (Radke et al. 2017, Wills et al. 2012). In order to knock down GluN2B, we used an intrathecal injection of AAV9 virus carrying the gene for Cre-recombinase driven by a CMV promoter. This approach enabled us to selectively knock down GluN2B largely restricted to spinal cord tissue.

Agmatine Modulates Opioid Analgesic Tolerance

In addition to agmatine's direct modulation of tactile hypersensitivity following injury, agmatine has also been shown to modulate the efficacy of opioid analgesia (Su, Li and Qin 2003) and prevent the development of opioid analgesic tolerance (Kolesnikov, Jain and Pasternak 1996, Fairbanks and Wilcox 1997) through indirect interaction with opioid receptors. This action occurs through their inhibition of NMDA receptors as previously discussed (Elliott et al. 1995), but also through agmatine's action as a competitive NOS inhibitor (Galea

et al. 1996). These observations support the development of agmatine as an adjunct therapeutic to be delivered alongside opioids to increase the therapeutic potential of opioids while reducing undesirable side effects of opioids including tolerance and dependence. Previous work has shown that agmatine given alone in doses of 0.1 or 10 mg/kg had no significant effect in a mouse tail flick assay (Kolesnikov et al. 1996). However, delivery of agmatine alongside morphine shifts morphine's ED_{50} 2-9 fold, depending on the route of morphine's administration (intracerebroventricularly (i.c.v.) and intrathecally (i.t.), respectively). As an extension of these results, daily i.c.v. injections of 10 nmol agmatine alone does not potentiate acute morphine analgesic potency compared to saline controls (Kitto and Fairbanks 2006). These results support the argument that agmatine's attenuation of morphine tolerance is not due to acute potentiation of morphine analgesia. It then becomes necessary to probe agmatine's impact on the development of morphine tolerance in mice, as well as the necessity of the 2B subunit of the NMDA receptor in lumbar spinal cord on this impact.

Materials & Methods

Animals

The GluN2B-floxed allele mouse was generated by the Gene-Targeted Mouse Core of the INIA-stress consortium, as previously described (Brigman et al. 2010). This Integrative Neuroscience Initiative on Alcoholism examines the

link between stress and alcohol. The consortium is supported by the National Institute on Alcohol Abuse and Alcoholism. The Gene-Targeted Mouse Core is supported by NIH grant U01 AA013514 to Eric Delpire.

Intrathecal injections

All drugs were dissolved in sterile saline and delivered in 5-µl volumes via intrathecal injection in conscious mice (Hylden and Wilcox 1980). Briefly, the mice were held by the iliac crest and a 30-gauge, 0.5 inch needle attached to a 50-μL Luer-hub Hamilton syringe delivered 5 μL of injectate into the intrathecal space of the mice.

Spared Nerve Injury

Tactile hypersensitivity was induced using the spared nerve injury model described by Decosterd and Woolf (Decosterd, Allchorne and Woolf 2002). Subjects are placed under isoflurane anesthesia and the left sciatic nerve is exposed, along with its three terminal branches. The common peroneal and tibial nerves were ligated with 5.0 silk suture. The nerves were sectioned 2 mm distal to the ligation site. The sural nerve remained uninjured.

Tactile Hypersensitivity

Mice were placed on a wire mesh grid under a glass enclosure and allowed to acclimate for 30 minutes prior to testing. Hypersensitivity was tested by using an electronic von Frey device (Life Sciences, IITC). The left and right hindpaws were stimulated by the tip of the stimulator with enough force to cause the mouse to withdraw its paw. The amount of force required for withdrawal was recorded in grams. Baseline responses before SNI were collected, and the %MPE was calculated by the following formula: (Experimental Value-Control)/(Cutoff-Control) X 100. For the purpose of these experiments, the following measurements were used: (Post-Drug Threshold – Pre-Drug Threshold)/(Pre-Surgery Baseline Threshold – Pre-Drug Threshold) X 100.

Induction of Morphine Analgesic Tolerance

Morphine tolerance was induced in mice by repeated administration of intrathecal morphine over the course of four days. Mice were assessed for their baseline nociceptive responsiveness on Day 1, followed by an intrathecal injection of 10 nmol morphine (control) or 10 nmol morphine + 10 nmol agmatine (experimental). On days 2, 3 and 4, mice received twice daily intrathecal injections and a tail flick assay following the second injection. All injections were delivered in a volume of 5 microliters.

Warm Water Tail Immersion

A warm water (52.5 °C) immersion tail-flick assay was used to assess nociceptive responsiveness (PA, CJ and JG 1963). Baseline measurements of tail-flick latencies were collected on every subject and each subject's baseline was used as its own control. The maximum possible effect (%MPE) was calculated as follows: %MPE = (postdrug latency – predrug latency) / (cutoff – predrug latency) x 100%. A maximum score of 100% was assigned to animals that did not show responsiveness before the 12-second cutoff to avoid tissue damage.

Generation of GluN2B-Deficient Mice

Generation of the GluN2B mouse was initiated by Dr. E. Delpire (Vanderbilt University), as previously described (Brigman et al. 2010). The GluN2B mutant mouse was generated by the Gene-Targeted Mouse Core of the INIA-stress consortium. This Integrative Neuroscience Initiative on Alcoholism examines the link between stress and alcohol. The consortium is supported by the National Institute on Alcohol Abuse and Alcoholism. The Gene-Targeted Mouse Core is supported by NIH grant U01 AA013514 (to E.D.). A breeding colony of homogenous GluN2B-floxed mice was established. At time of weaning (p21), all subjects received either an intrathecal injection of 5 microliters of 0.9% saline or AAV9.CMV.HI.eGFP-Cre.WPRE.SV40 (Penn Vector Core, University of Pennsylvania).

RT-qPCR Confirmation of GluN2B-Deficiency

Lumbar spinal cord tissue was collected in TRIzol® Reagent (phenol and guanidine isothiocyanate solution) in order to confirm the genotype of each subject. Total RNA was extracted according to the manufacturer's instructions. The RNA pellet was dissolved in nuclease-free water and RNA concentration was estimated by spectrophotometric analysis using the NanoDrop®ND-1000 Spectrophotometer (Thermo Fisher Scientific). An equal amount of RNA was used for each reaction.

The expression levels of NMDA receptor subunits GluN2A and GluN2B were determined by estimating the messenger RNA copy number through quantitative real-time reverse transcription (RT)-PCR method. All reactions were set up in 96-well format (Multiplate™ 96-Well PCR Plates, BIO-RAD) and carried out in CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD) using iTaq^{TM} Universal SYBR® Green One-Step Kit (BIO-RAD). The oligonucleotide primers used were; Mouse GluN2A: F 5'-TCTATGACGCAGCCGTC TTGAACT-3' and R-5'-TGTGGTAGCAAAGATGTACC CGCT-3', GluN2B: F 5'-ATG AAGAGGGGCAAG GAGTT-3' and R 5'-CG ATG ATGGAGGAGACTTGG-3', 18S F 5'-AAGACGATCA GATACCGTCGTAG-3' and R 5'-TCCGTCAATTCCTTTAAG TTTCA-3' (Dhar and Wong-Riley 2009, Tajerian et al. 2015). All reactions were run in triplicate. Each 20 μl of reaction mixture contained 10µl, 2X master mix, 0.25 µl enzyme mix, 300nM of each primer, and 40ng of RNA. Two no template control (NTC) wells were included in each run. Wells were sealed with optically

clear film. The PCR cycling conditions were 20 min at 50ºC, 1min at 95ºC and then 45 cycles each of denaturation at 95ºC for 10s and annealing and extension at 60ºC for 30s. Melting curve analysis was performed to ensure the amplification of a single product in each reaction. Amplification data were analyzed by CFX Manager software version 3.1 (BIO-RAD). Data (Ct values) were analyzed using a comparative Ct method (ΔΔCt method (Schmittgen and Livak 2008). Endogenous control, 18S was used; as it has been validated as a stable normalization gene for RT-qPCR (Piller, Decosterd and Suter 2013). To obtain the ΔCt value for each of the sample the Ct value of 18S was subtracted from the Ct value of target (GluN2A/2B). The ΔΔCt was obtained by using the ΔCt experimental value (AAV9-cre injected) minus the ΔCt control value (saline injected). Then the fold change ($2^{-\Delta\Delta Ct}$) was calculated.

Data Analysis

All statistical analysis was considered significant at $\alpha = 0.05$. Mechanical paw withdrawal thresholds collected by von Frey filament stimulation were analyzed by repeated measures ANOVA with Bonferroni post-hoc corrected analysis. Analysis of RT-qPCR was performed by normalizing the data to a housekeeping gene, 18S, and compared by unpaired Student's t-test. For induction of morphine tolerance, an unpaired Student's t-test was performed on maximum possible effect (%MPE) between experimental and control groups.

Results

Generation of Temporally- and Site-Restricted Reduction of GluN2B

At time of weaning (p21), Grin2B-floxed mice were intrathecally injected with either saline or AAV9-cre to generate wild type or GluN2B-deficient mice, respectively. A minimum of two weeks following injection, lumbar spinal cord tissue was collected and analyzed by RT-qPCR for Grin2B and Grin2A expression in both wild type and GluN2B-deficient mice. We observed a significant decrease in Grin2B, but not Grin2A, mRNA expression in lumbar spinal cord, as is represented in Figure 1.

Agmatine Requires GluN2B-Containing NMDA Receptors to Attenuate Neuropathic Pain

The first aim of this study was to determine whether GluN2B is required for agmatine's demonstrated reversal of pain behaviors. Two GluN2B-floxed cohorts were run in parallel, one that had received an injection of saline at time of weaning (wild type) and another that received an injection of AAV9-cre at time of weaning (GluN2B KD) (Table 2). A minimum of 4 weeks following this injection, every subject in both cohorts received spared nerve injury (SNI) to establish a state of chronic neuropathic pain. All subjects were assessed for their von Frey thresholds prior to surgery and alternating days after surgery (days 1, 3, 5, and 7) on both the injured (ipsilateral) and non-injured (contralateral) hindpaws. Agmatine (10 nmol) or saline was delivered intrathecally prior to surgery and on

days 2, 4, and 6 post-surgery. Additional von Frey testing continued weekly for a maximum of 30 days following injury, as is represented in Figure 2. Following completion of behavioral testing, spinal cords were extracted and analyzed for GluN2A and GluN2B levels. We observed that nerve-injury reduced pain thresholds in both GluN2B-deficient (Figure 2A) and wild type (Figure 2B) mice. During the induction phase (first post-operative week, intrathecal treatment of agmatine had little effect in either group. During the maintenance phase (period of established chronic pain) agmatine demonstrated no effect in the GluN2B-deficient mice whereas it reduced tactile hypersensitivity in the wild type mice, consistent with agmatine's effects in ICR mice shown in Chapter 2. These results suggest that the anti-hyperalgesic effects of agmatine require the GluN2B subunit of the NMDA receptor

MK-801 Does Not Require the GluN2B Subunit of the NMDA Receptor to Reduce Neuropathic Pain Behavior

Based on agmatine's lack of efficacy in GluN2B-deficient mice in the SNI model of neuropathic pain, we expanded this work to include additional, gold standard NMDA antagonists. MK-801 is established to bind in the open channel of NMDA receptors (Huettner and Bean 1988, Wong et al. 1986), and as such should not require the presence of GluN2B in order to have efficacy in reversing pain behaviors. Two GluN2B-floxed cohorts were run in parallel, one that received an i.t. injection of saline (wild type) at time of weaning and another that

received i.t. AAV9-cre (GluN2B-deficient) (Table 3). All subjects were behaviorally assessed prior to SNI and days 1, 3, 5, and 7 following SNI (Figure 3). Following completion of behavioral testing, spinal cords were extracted and analyzed for Grin2A and Grin2B levels. We observed moderate efficacy of MK-801 in both the wild type and GluN2B-deficient mice at reversing long-term hypersensitivity following SNI. We observed that, like agmatine, MK-801 had little effect in either GluN2B-deficient or wild type nerve-injured mice during the induction phase of tactile hypersensitivity. However, during the maintenance phase, MK-801 demonstrated reduces tactile hypersensitivity in the GluN2B-deficient mice even more robustly in the wild type mice. These data suggest that, unlike agmatine, the GluN2B receptor subunit is not required for MK-801's anti-hyperalgesic effects in nerve-injured mice.

Ifenprodil Requires GluN2B-Containing NMDA Receptors to Attenuate Neuropathic Pain

Ifenprodil has been demonstrated to be selective for the 2B subunit of the NMDA receptor (Gallagher et al. 1996, Chenard and Menniti 1999, Williams 2001). As such, we sought to parallel agmatine's lack of efficacy in GluN2B-deficient mice with ifenprodil. Two GluN2B-floxed cohorts were run in parallel, one that had received an injection of saline at time of weaning (wild type) and another that received an injection of AAV9-cre at time of weaning (GluN2B KD) (Table 4). A minimum of 4 weeks following this injection, every subject in both

cohorts received SNI to establish a state of chronic neuropathic pain. All subjects were assessed for their von Frey thresholds prior to surgery and alternating days after surgery (days 1, 3, 5, and 7) on both the injured (ipsilateral) and non-injured (contralateral) hindpaws. Additional von Frey testing continued weekly for a maximum of 30 days following injury (Figure 4). Following completion of behavioral testing, spinal cords were extracted and analyzed for Grin2A and Grin2B levels. We observed that, like in the agmatine and MK-801 experiments, ifenprodil has no effect during the induction phase of tactile hypersensitivity. Similar to agmatine, during the maintenance phase ifenprodil showed no effect in the GluN2B-deficient mice but did reduce tactile hypersensitivity in the wild type mice, consistent with ifenprodil's effects in ICR mice shown in Chapter 2.

Agmatine Requires the GluN2B Receptor Subunit to Prevent the Development of Morphine Analgesic Tolerance

In addition to assessing the requirement of the GluN2B subunit for agmatine's reduction of neuropathic pain, we studied the effects of GluN2B knock down on agmatine's well established ability to prevent the development of spinal opioid tolerance. We hypothesized that, like the requirement of GluN2B for agmatine's inhibition of neuropathic pain, GluN2B would be required for agmatine's effects on spinal opioid analgesic tolerance. Chronic pain and opioid analgesic tolerance have long been known to share neuroplasticity-related

mechanisms based on NMDA-receptor activation (Mayer et al. 1999). Two GluN2B-floxed cohorts were run in parallel, one that received a saline injection at time of weaning, and one that received AAV9-cre at time of weaning (Table 6). A minimum of 4 weeks following this injection, subjects were injected daily with either morphine of morphine + agmatine and assessed for their tail-flick latencies (Table 5). Following the 4th injection, mice were assessed for their tail flick latency and the percent efficacy was assessed (Figure 5). In Figure 5A, the analgesic responses to morphine on day 1, 2, 3, and a final day 4 are displayed for subjects that received either the morphine tolerance-inducing dose or that same dose + agmatine. It is clear that those that receive morphine progressively reduces analgesic efficacy by the third day whereas the group that received agmatine maintains analgesia at a constant level throughout the testing period. This pattern is consistent with prior studies demonstrating that agmatine protects against the development of morphine tolerance. In contrast, in the GluN2B-deficient mice (Figure 5B), both morphine-treated and morphine + agmatine treated mice demonstrate rapid and dramatic losses of morphine efficacy by Day 3. In this population, agmatine was ineffective. The magnitude of tolerance induction was assessed for each treatment group and represented in Figure 5C where it is shown that in the WT mice the magnitude of tolerance development is greatly diminished in the “Morphine + Agmatine” group relative to “Morphine alone”; there is no difference in the magnitude of tolerance development in the GluN2B-deficient mice. These data support the hypothesis that GluN2B is

required for the protective effect of agmatine in the development of opioid tolerance.

Figure 1

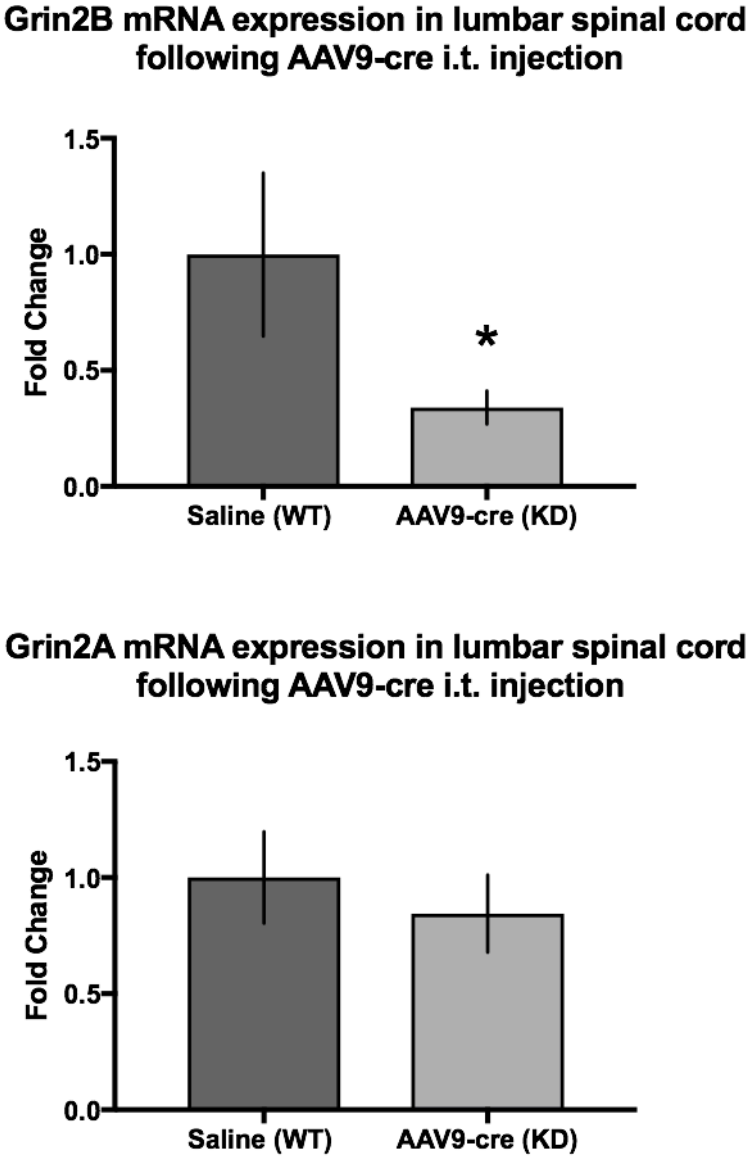

Figure 1: Analysis of Grin2B reduction in GluN2B-floxed mice following AAV9-cre injection. At time of weaning, subjects were injected with either saline control (WT) or AAV9-cre (KD). A minimum of two weeks following injection, lumbar spinal cord was collected and analyzed by RT-qPCR for Grin2B and Grin2A mRNA expression. Unpaired Student's t test, * represents significant difference from saline control $p < 0.05$.

Table 1: Study Timeline of NMDA-Mediated Inhibition of Maladaptive Neuroplasticity Following SNI

Day -1	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**
von Frey (vF) threshold baseline	i.t. injection of 10 nmol study drug or saline SNI	vF Threshold	i.t. 10 nmol study drug	vF Threshold	i.t. 10 nmol study drug
Day 5	**Day 6**	**Day 7**	**Day 26**	**Day 31**	**Day 32**
vF Threshold	i.t. 10 nmol study drug	vF Threshold	vF Threshold	vF Threshold	Tissue extraction for mRNA analysis of Grin2B and Grin2A levels

Table 2: Study Composition of i.t. Agmatine

or Saline Delivery Following SNI

	Pre-Treatment i.t. Saline (4 weeks prior to experiment)	**Pre-Treatment i.t. AAV9-CMV-cre (4 weeks prior to experiment)**
i.t. 10 nmol Agmatine	9 mice (6 M, 3 F)	9 mice (6 M, 3 F)
i.t. Saline	8 mice (8 M)	9 mice (7 M, 2 F)

Figure 2

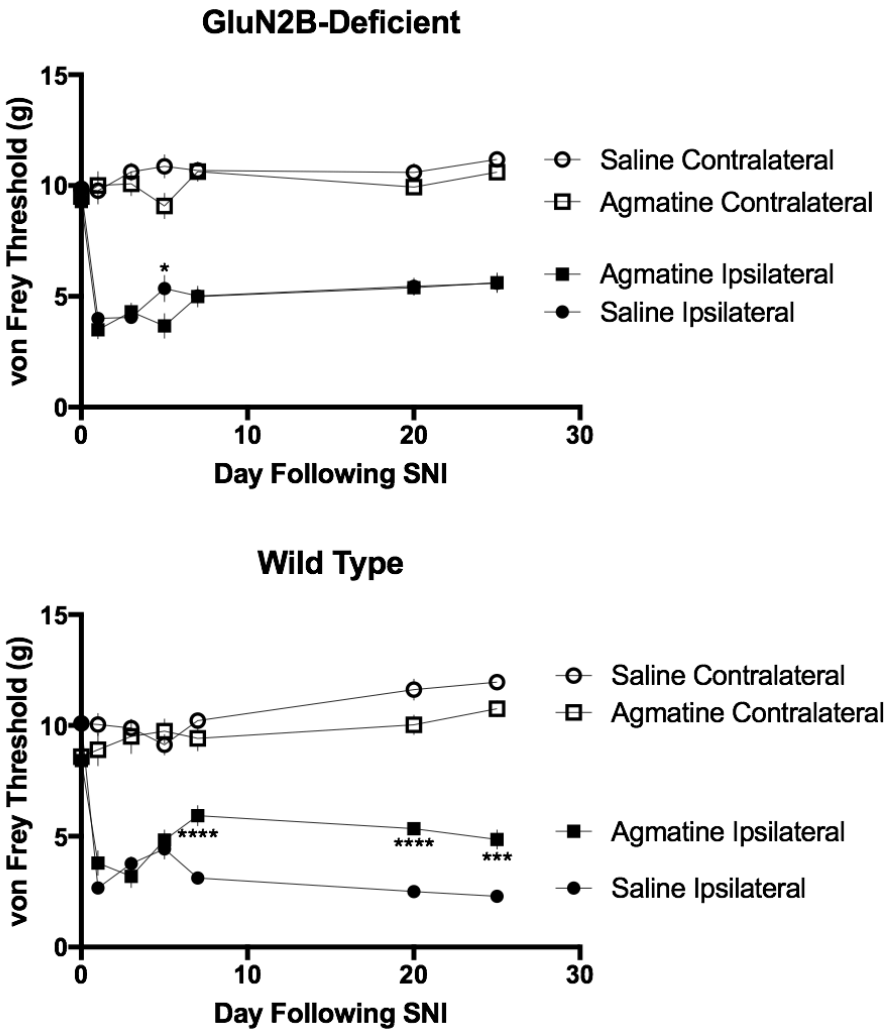

Figure 2: Intrathecal agmatine attenuates neuropathic pain responses in wild type, but not GluN2B-deficient mice. Two cohorts (wild type control and GluN2B-deficient) were run in parallel. All subjects were given spared nerve injury to induce local hypersensitivity. Immediately prior to surgery, subjects received saline control or 10 nmol agmatine, i.t. Subjects also received saline or agmatine on days 2, 4, and 6 following injury. von Frey thresholds were taken prior to and following injury. * represents significant difference from saline control. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ANOVA with Bonferroni post-hoc analysis.

Table 3: Study Composition of i.t. MK-801

or Saline Delivery Following SNI

	Pre-Treatment i.t. Saline (4 weeks prior to experiment)	**Pre-Treatment i.t. AAV9-CMV-cre (4 weeks prior to experiment)**
i.t. 10 nmol MK-801	7 mice (5 M, 2 F)	10 mice (6 M, 4 F)
i.t. Saline	8 mice (2 M, 6 F)	10 mice (8 M, 2 F)

Figure 3

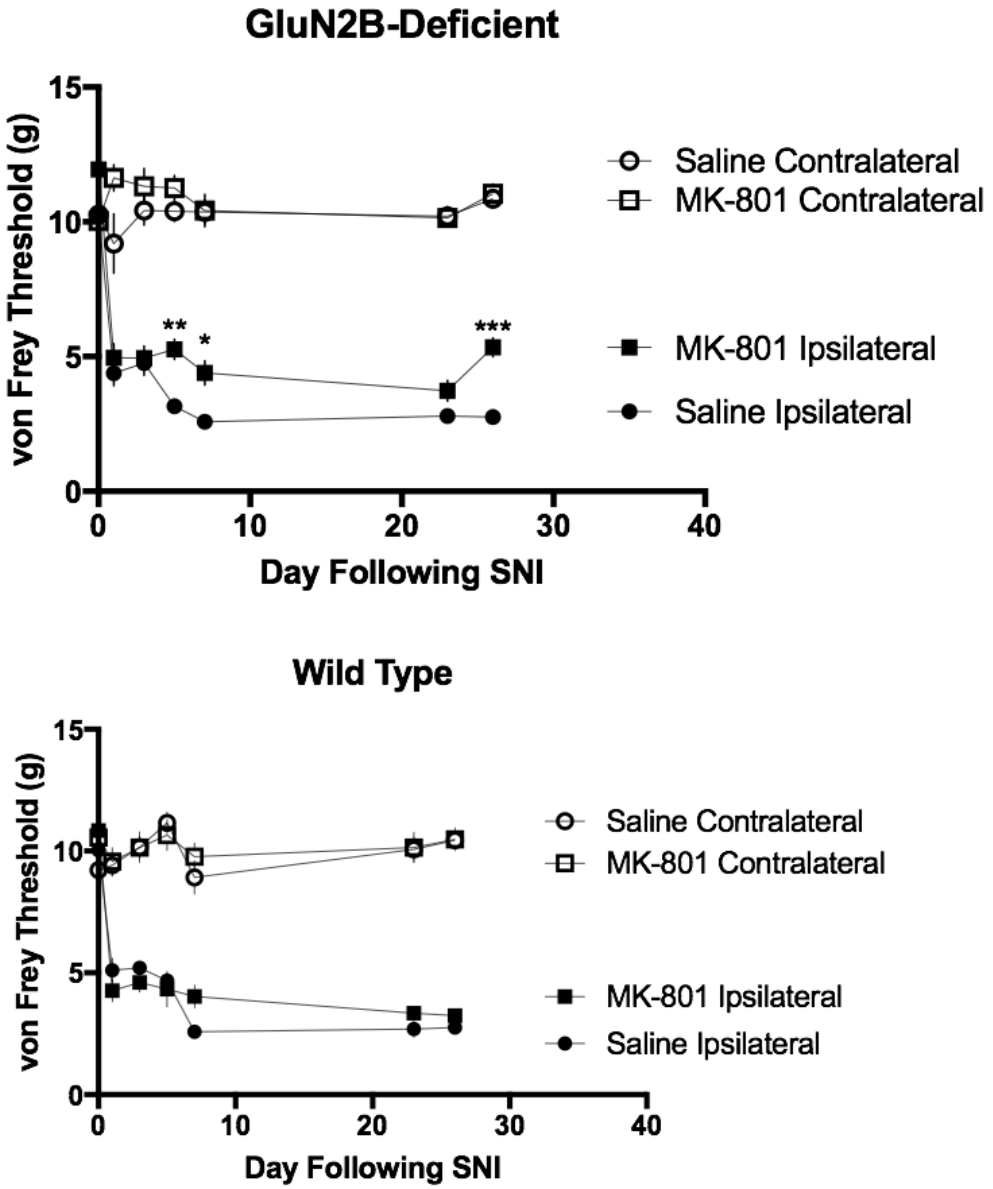

Figure 3: MK-01 attenuates neuropathic pain behaviors. Two cohorts (wild type control and GluN2B-deficient) were run in parallel. All subjects were given spared nerve injury to induce local hypersensitivity. Immediately prior to surgery, subjects received saline control or 10 nmol MK-801, i.t. Subjects also received saline or MK-801 on days 2, 4, and 6 following injury. von Frey thresholds were taken prior to and following injury. * represents significant difference from saline control. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ANOVA with Bonferroni post-hoc analysis.

Table 4: Study Composition of i.t. Ifenprodil

or Saline Delivery Following SNI

	Pre-Treatment i.t. Saline (4 weeks prior to experiment)	**Pre-Treatment i.t. AAV9-CMV-cre (4 weeks prior to experiment)**
i.t. 10 nmol Ifenprodil	9 mice (5 M, 4 F)	9 mice (4 M, 5 F)
i.t. Saline	9 mice (6 M, 3 F)	9 mice (4 M, 5 F)

Figure 4

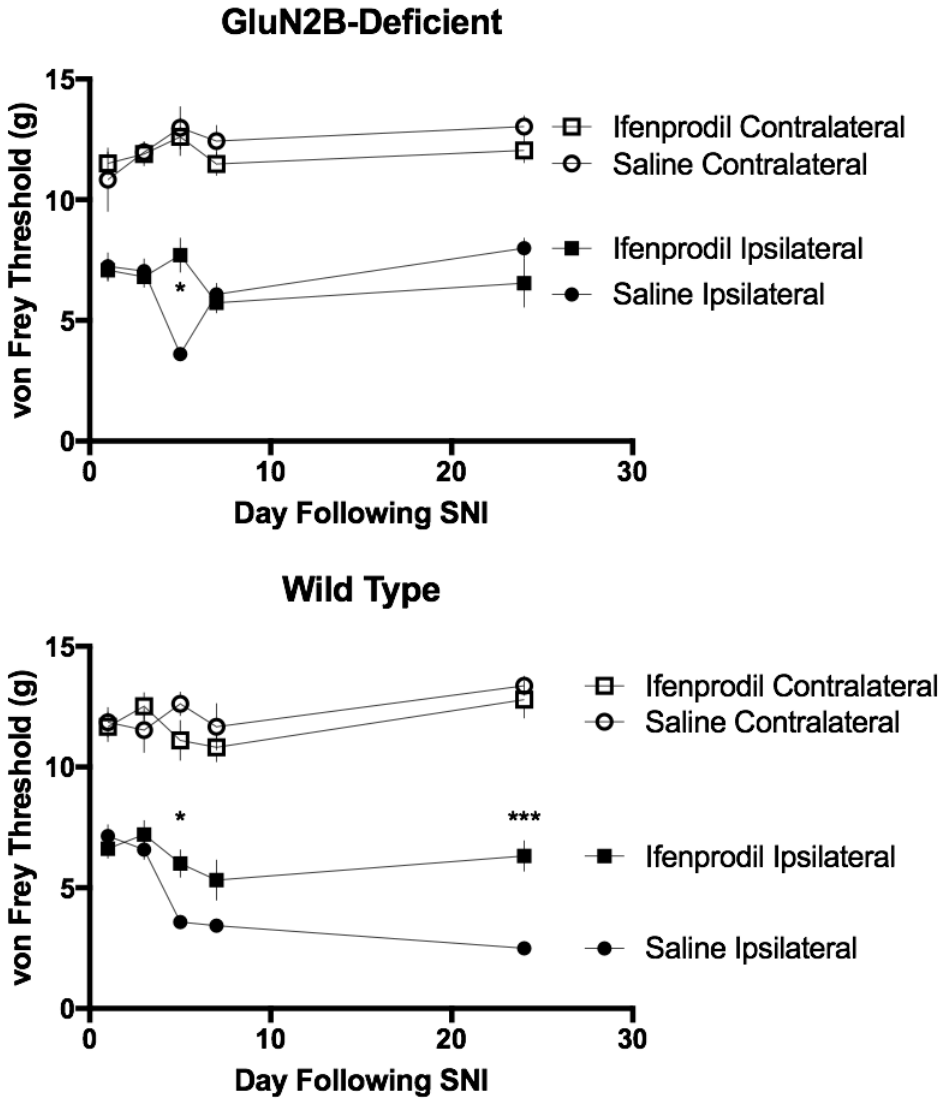

Figure 4: Intrathecal ifenprodil attenuates neuropathic pain responses in wild type, but not GluN2B-deficient mice. Two cohorts (wild type control and GluN2B-deficient) were run in parallel. All subjects were given spared nerve injury to induce local hypersensitivity. Immediately prior to surgery, subjects received saline control or 10 nmol ifenprodil, i.t.. Subjects also received saline or ifenprodil on days 2, 4, and 6 following injury. von Frey thresholds were taken prior to and following injury. * represents significant difference from saline control. $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ANOVA with Bonferroni post-hoc analysis.

Table 5: Study Timeline of Morphine Tolerance in GluN2B-Deficient or Wild Type Mice

Day 1	Day 2	Day 3	Day 4	Day 5
AM: N/A	AM: 10nmol i.t. MS or MS + AG	AM: 10nmol i.t. MS or MS + AG	AM: 10nmol i.t. MS or MS + AG	AM: Tissue extraction for confirmation of Grin2B knock down
PM: Baseline Tail Flick 10nmol i.t. morphine sulfate (MS) or morphine sulfate + agmatine (MS + AG)	PM: 10nmol i.t. MS or MS + AG Tail Flick	PM: 10nmol i.t. MS or MS + AG Tail Flick	PM: 10nmol i.t. MS or MS + AG Tail Flick	

Table 6: Study Composition of Agmatine Attenuation of Morphine Tolerance in Wild Type or GluN2B-Deficient Mice

	Pre-Treatment i.t. Saline (4 weeks prior to experiment)	**Pre-Treatment i.t. AAV9-CMV-cre (4 weeks prior to experiment)**
i.t. 10 nmol Morphine Sulfate	8 mice (4 M, 4 F)	11 mice (4 M, 7 F)
i.t. 10 nmol Morphine Sulfate + Agmatine	8 mice (3 M, 5 F)	11 mice (6 M, 5 F)

Figure 5

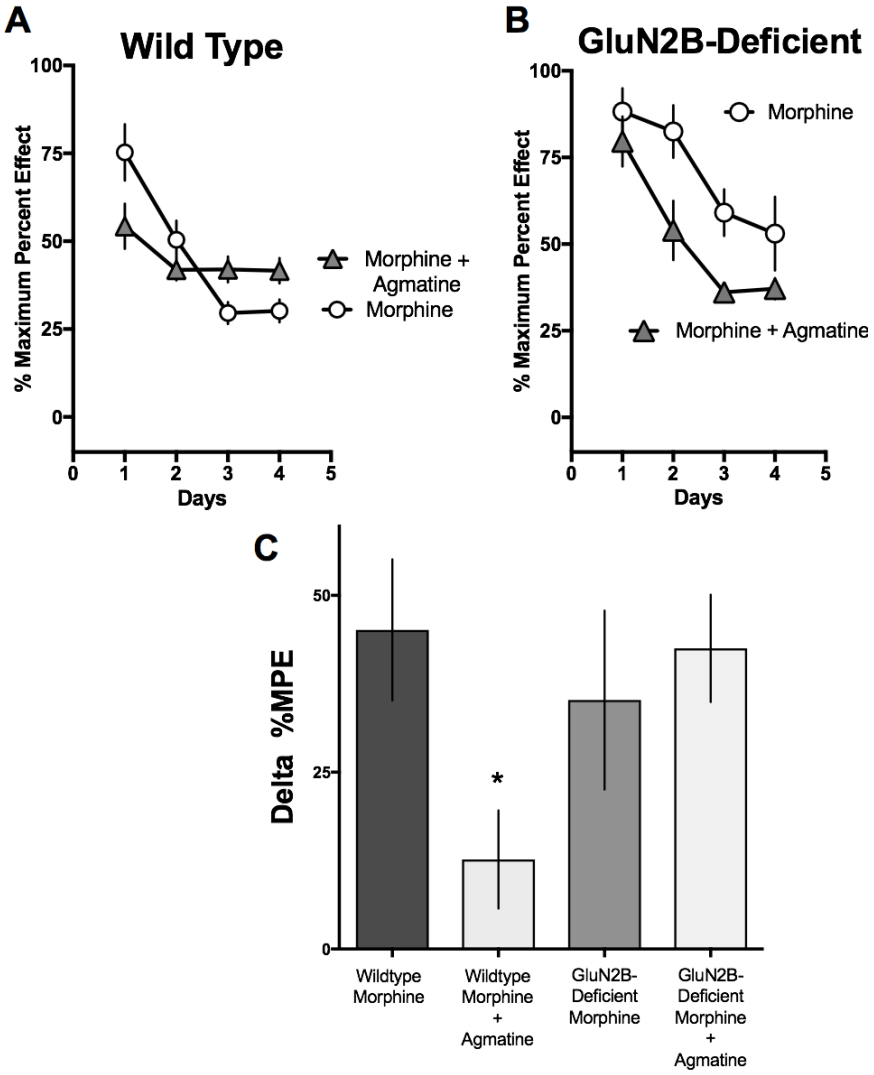

Figure 5: Agmatine attenuation of morphine tolerance in wild type but not GluN2B-deficient mice. Two cohorts (A – Wild Type control and B - GluN2B-Deficient) were run in parallel. All subjects received either morphine or morphine + agmatine, i.t. twice daily for four days and were assessed for their tail flick latency prior to induction of tolerance and following daily morphine dosing. C) The maximum percent effect (%MPE) was calculated for both day 1 analgesic effects following morphine and day 4 analgesic effects following probe morphine. This difference was calculated and is represented as delta %MPE. Unpaired Student's t test within group, * represents significant difference from the wild type morphine-only control $p < 0.05$.

Discussion

The first major finding of these studies was the development of a viable knock down of the GluN2B subunit of the NMDA receptor in lumbar spinal cord while avoiding the characterized side effects of a global knockout from birth. Figure 1 demonstrates the decrease in spinal GluN2B 4 weeks following the injection of AAV9-cre into GluN2B-floxed weanlings, leading to a temporally- and anatomically-restricted reduction.

We then tested the hypothesis that agmatine requires the 2B subunit of the NMDA receptor following intrathecal delivery, and that this interaction is responsible for agmatine's efficacy in inhibiting the expression of neuropathic pain behaviors *in vivo* following spared nerve injury. We utilized the same model of inhibition of maladaptive neuroplasticity described in Chapter 2 and compared agmatine's efficacy at reducing the expression of neuropathic pain in wild type and GluN2B-deficient mice when given intrathecally immediately prior to spared nerve injury and alternating days following injury. As is consistent with polyamines preferring GluN2B subunits of the NMDA receptor, agmatine was not efficacious at inhibiting the induction of pain behaviors in mice with lower levels of GluN2B as compared to wild type mice (Figure 2). This effect persisted for a month following injury, at which time the lumbar spinal cord was harvested for confirmation of GluN2B deficiency.

In order to parallel our work described in Chapter 2, we conducted this study of inhibition of maladaptive neuroplasticity with ifenprodil in both GluN2B-

deficient and wild type mice. As was expected, intrathecally-delivered ifenprodil was unable to robustly inhibit the development of maladaptive neuroplasticity in GluN2B-deficient mice, but was efficacious in wild type controls (Figure 4). These results give strength to the hypothesis that agmatine, like ifenprodil, preferentially acts at GluN2B-containing NMDA receptors in the dorsal horn of the spinal cord. We sought to perform another pharmacological control in this model using MK-801, a channel-blocker not reliant on the GluN2B subunit of the NMDA receptor, again as a parallel to the studies performed in Chapter 2, and found little appreciable difference between GluN2B-deficient and wild type controls.

The behavioral data presented here concerning agmatine's protection from the development of morphine tolerance did reach significance in wild type, but not in GluN2B-deficient, mice (Figure 5). This is indicative of agmatine requiring GluN2B containing NMDA receptors in order to inhibit the development of opioid tolerance. This paradigm has been studied and published previously with the same experimenter and injector (Kitto and Fairbanks 2006) and has been repeated multiple times (Churchill 2014), but this is its first evaluation in GluN2B-deficient mice.

Summary and Conclusion

The data presented here support the hypothesis that agmatine preferentially acts at the GluN2B subunit of the NMDA receptor. Electrophysiological (Wataaja, in preparation), behavioral, and molecular data all support this mechanism of action. Additionally, agmatine requires this subunit to inhibit maladaptive plasticity induced by chronic neuropathic pain or opioid tolerance. This characteristic of agmatine makes it an attractive and viable candidate for translation to clinical use.

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Chapter 5: Summary, Future Directions, and Conclusion

Summary

The research described in this thesis has shown the efficacy of exogenous agmatine (Chapter 2) and gene therapy-induced increase of endogenous agmatine (Chapter 3) at reducing the expression of hypersensitivity via inhibition of the GluN2B subunit of the NMDA receptor (Chapter 4). These findings indicate the value of development of agmatine therapy (whether exogenous delivery or gene therapy) for clinical application, including directly for relief of pain and indirectly as an opioid adjunct to prevent the development of opioid tolerance. This work points to the necessity of understanding the long-term effects of gene therapy and elevated agmatine.

Conclusions

Agmatine Clinical Safety and Efficacy

Agmatine remains a novel and exciting prospective anti-hyperalgesic for chronic pain treatment due to its endogenous synthetic and degradative pathways, making its synthesis an attractive gene therapy target, as well as its current availability as a nutraceutical (Neis et al. 2017). Long term safety studies of orally delivered agmatine have been completed in rats (Gilad and Gilad 2013) and humans (Gilad and Gilad 2014), including a randomized, double-blind, placebo-controlled trial of oral agmatine safety and efficacy in lumbar disc-associated radiculopathy (Keynan et al. 2010). In the dose escalation safety study, participants received 1.335 – 3.560 g/day over a period of 40-45 days and were interviewed 6 months after initiation of treatment to evaluate the safety of this regimen. Three participants in the safety study reported mild-to-moderate diarrhea and nausea that ceased after agmatine was halted. In the placebo-controlled trial, agmatine was significantly more efficacious than placebo at relieving pain from lumbar disc herniation as measured by scores on the McGill Pain Questionnaire and the visual analog scale. These data begin to establish the safety and efficacy of long-term agmatine use in a clinical population.

Future Directions

Development and Analysis of Strategically-Substituted Agmatine (SSA) Compounds

Agmatine is a polyamine transmitter; it is rapidly metabolized in the periphery and has limited penetration into the central nervous system (CNS). Additionally, the half-life of agmatine given systemically is 30 minutes (Roberts et al. 2005). The studies presented in this thesis utilized the intrathecal (i.t.) route of delivery in order to analyze exogenously delivered agmatine's actions at the spinal cord, but a more accessible route of delivery must be considered for agmatine to be a clinically useful and relevant therapy. To this end, we have previously developed and begun characterization of a line of strategically-substituted agmatine (SSA) compounds designed to improve their penetration into the CNS and half-life, thus increasing their suitability for use as pharmacological agents. We have begun testing these agents in models of peripheral hypersensitivity following nerve injury, respiratory depression, fear-conditioned memory recall, and motor coordination. We have also begun analyzing their pharmacokinetic profile compared to agmatine in order to identify promising SSAs for further analysis and translation.

Agmatine Inhibition of Self-Administered Opioid Acquisition

A pharmacological blockade of NMDA receptors has previously been shown to inhibit the acquisition of conditioned place preference (CPP), a model of

the early stages of addiction (Kalivas and Volkow 2011). Agmatine has been demonstrated to inhibit the N-methyl-D-aspartate receptor without known side effects of NMDA inhibition including motor impairment and inhibition of learning and memory, as discussed in Chapter 2 and Chapter 3, making it a promising candidate for adjunct opioid therapy. Previous work with agmatine and SSAs has shown the efficacy of these compounds in providing analgesia in various pain models including neuropathic and inflammatory pain (Yu et al. 2000, Fairbanks et al. 2000). Agmatine has also been shown to be effective at attenuating the development of oral fentanyl self-administration (Wade et al. 2008). However, because agmatine is a polyamine transmitter, it is rapidly metabolized in the periphery and has limited penetration into the CNS.

In order to address this, we have begun using targeted injections of AAV vectors designed to express arginine decarboxylase (ADC), thus increasing endogenous agmatine in a sustained and site-specific manner at key points along the glutamatergic reward pathway. We have also begun developing and characterizing a line of SSAs designed to improve their penetration into the CNS and their half-life, thus increasing their suitability for use as an inhibitor of opioid addiction acquisition following systemic injection. We will test the hypothesis that agmatine is an effective inhibitor of the acquisition of oxycodone self-administration using these two strategies. Development of adjunct therapy to the delivery of traditional opioids allows continuation of use of well-characterized, clinically available analgesics such as fentanyl and oxycodone while attenuating

their addictive potential. Through these studies, we aim to comprehensively characterize an adjunct therapy to maximize the analgesic potential of the most clinically relevant opioids while reducing potential side effects through dose reduction.

Conclusion

The National Institute of Medicine estimates that over 116 million Americans have or will experience chronic pain conditions in their lifetime (2016). However, most clinically available pain therapeutics fail to adequately manage patient's' symptoms and doctors are wary to prescribe available opioid formulations due to concerns about diversion, misuse, addiction, and overdose (Kroenke and Cheville 2017). The financial and societal impacts of chronic pain are monumental, with costs reaching over $635 billion per year in medical treatments and lost productivity. We critically need new, non-opioid and opioid-adjunct therapies to safely relieve the burden of chronic pain from pain patients. In this thesis I have described both small molecule and gene therapy approaches to managing chronic neuropathic pain. While further development and testing is needed, it is hoped that the research presented here will further the goal of developing safe and effective therapeutics for pain patients.

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