THE IMPACT OF GENE TRANSFER OF ARGININE DECARBOXYLASE TO THE CENTRAL NERVOUS SYSTEM ON OPIOID ANALGESIC TOLERANCE

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Chapter 1: INTRODUCTION

1.1 *Chronic Pain*

The National Institute of Medicine estimates that over 116 million Americans have one or more chronic pain conditions that result in medical treatments and loss of productivity estimating $635 billion or more each year (IOM Report, 2011). The most effective treatment available for chronic pain management remains opioid pharmacotherapy (Gintzler and Chakrabarti, 2008). However, opioids often fail to achieve all the therapeutic goals for eliminating or significantly reducing the patient's discomfort. It has been reported that only 20% of those using opioids for pain find them to be an effective, long lasting treatment (IOM Report, 2011). This is due in part to the fact that patients often have two or more causes or sources of their pain (Foley, 2000). There are also concerns with the patient and the prescriber for the possibility of opioid abuse and addiction. Other limiting side effects include reduction in sensory thresholds and development of opioid tolerance following long term exposure. It is clear that alternative strategies for pain management are greatly needed. This introduction will briefly review the current standard pharmacological treatments for chronic pain and opportunities for alternative approaches. This content establishes the foundation for the overall objective of the work featured in this thesis.

1.2 *Pharmacological Treatments for Chronic Pain*

Great effort and collaboration has taken place between researchers, clinicians and organizations like the American Pain Society (APS) and the International Association for

the Study of Pain (IASP) to create clinical guidelines for non-malignant chronic pain. Currently, the guidelines recommend a, "multi-modal analgesic technique" when designing a chronic pain treatment regime for patients (Joshi, 2005). In 1986, the World Health Organization (WHO) developed a standard treatment protocol for practitioners to follow when treating pain termed the WHO Analgesic Ladder. These guidelines were initially established as a common framework for treatment of post-operative or cancer-related pain. However, a similar strategy has been adopted when treating non-malignant chronic pain. Level 1 of the Analgesic Ladder describes appropriate treatment approaches for mild pain, which includes the use of non-opioid therapy such as acetaminophen, aspirin, and other Non-Steroidal Anti-Inflammatory Drugs (NSAIDS). Adjuvant therapy may also be included at this level. If pain persists or intensifies, the treatment is taken to the second level for mild or moderate pain. Medications appropriate for this level include those traditionally considered milder opioids such as tramadol or codeine. At Level 2, adjuvants can be given in addition to opioids, but they must be non-opioid adjuvant therapies. Level 3 is intended to address severe pain with stronger opioids such as fentanyl, morphine, oxycodone, or methadone either alone or together with adjuvants. If at any point in this process toxicity or side effects emerge, the dosage is reduced and/or opioid rotation implemented.

The Analgesic Ladder has served as an effective unifying framework for pain management since 1986. As we progress in our understanding of pain mechanisms and pathology, advances in treatment technologies have called for revision of the ladder (Vargas-Schaffer, 2010). One modification includes building a fourth step of the

Analgesic Ladder in order to incorporate more recently introduced interventional approaches when strong opioids become ineffective or to treat breakthrough pain crises. These strategies include, but may not be limited to implantation of epidural or intrathecal cathethers, transforaminal epidural steroid injections, neurosurgical interventions such as nerve blocks, or implantation of neurostimulation devices. It is likely that gene therapies, such as those proposed in this thesis, would fit on Level 4 of the Analgesic Ladder.

As the pharmacological, physiological, and sociological complexity of managing chronic pain, particularly with opioid medication, is increasingly recognized, the Analgesic Ladder may evolve such that alternative or more invasive therapies are not relegated to Level 4 but perhaps used in combination and as an adjuvant to Steps 1 and 2. The present thesis characterizes a gene therapeutic strategy that could be used in combination with opioid medication to 1) prevent the development of opioid-induced analgesic tolerance (and potentially addiction) and 2) to reduce dose requirements. The next few sections, will provide a brief review of the opioid receptor analgesic system and the NMDA receptor system, which are the receptor targets for the proposed combined gene therapy + opioid pain management strategy.

1.2.1 *Opioids*

As mentioned in the Introduction, the most effective treatment currently available for chronic pain sufferers remains opioid pharmacotherapy (Gintzler and Chakrabarti, 2008). Yet, opioids often fail to achieve all the therapeutic goals for eliminating or significantly reducing the patient's discomfort and are associated with many adverse

side-effects. Opioids act in the central nervous system (CNS) at G protein-coupled receptors (GPCRs). Ninety-percent of GPCRs (excluding odorant and light receptors) are expressed in the CNS, with nearly 80% of the identified G protein coupled-receptor (GPCR) families play a role in pain modulation (Stone, 2009). Opioid receptors are G protein-coupled receptors that are located throughout the CNS with regionalization in areas relevant to the sensory pain pathway (e.g. spinal cord dorsal horn, rostral ventral lateral medulla, periaqueductal gray). This feature makes GPCRs ideal candidates for pharmaceutical applications involving chronic pain. A decades-old and intensive, ongoing investigation by academia and industry has attempted to optimize pharmacotherapy targeting inhibitory GPCRs (Gi) with limited success, especially in regards to chronic pain management (Wess, 2007).

As this relates to pain signaling, the general mechanism of action for opioids is by activating both pre- and post-synaptic opioid receptors to inhibit pro-nociceptive signaling. The Gi protein has three subunits ($\alpha\beta\gamma$) that dissociate from one another and then act on various distinct downstream pathways. $G\alpha$ primarily works by inhibiting adenylyl cyclase, therefore reducing cAMP production, which in turn reduces activation of PKA. $G\beta\gamma$ subunits interact with the potassium ion channels in the post-synaptic neuron to increase intracellular potassium flow out of the cell resulting in hyperpolarization, which inhibits the neuron from carrying forward a signal, in this case, pain. $G\beta\gamma$ subunits also act to block voltage gated calcium channels in the pre-synaptic neuron to reduce neurotransmitter release. Both of these actions result in prevention of the pain signal from being transmitted to the brain and interpreted as pain. Therefore,

when an opioid, such as morphine is given, these GPCR pathways are set in motion and analgesia ensues.

Yet, even after 40 years of opioid research, opioid therapy for pain often fails to achieve all the therapeutic goals for eliminating or significantly reducing the patient's discomfort. This is mostly due to the fact that opioids are accompanied with a variety of adverse systemic side effects such as: sedation, dizziness, nausea, constipation, and risk of respiratory depression. Additionally, in the case of chronic use or chronic exposure to opioids, some individuals can experience opioid-induced hyperalgesia, where the opioid increases pain sensation.

Most relevant to the research presented here is the phenomenon of opioid-induced tolerance. Pharmacologically defined, tolerance is a statistically significant shift to the right in the dose-response curve. To a patient, this means their dose of opioid prescribed no longer results in adequate pain relief, resulting in an increase of the prescribed dose. The mechanisms involved in opioid tolerance have been described as a "moving target," where the multiple points of cellular adaptions arise to compensate for chronic inhibition of excitatory input (Gintzler and Chakraabarti, 2008).

1.2.2 *NMDA Receptor Antagonists as a Therapeutic Target for Preventing Opioid Analgesic Tolerance*

The N-methyl-D-aspartate receptor (NMDA) is an ion channel expressed in the postsynaptic membrane that is important in the process of synaptic plasticity, which drives much of neuroadaptation. The neurotransmitter glutamate is released from the

pre-synaptic neuron to communicate information, such as sensory stimulation (e.g. pain). Glutamate crosses the synapse and binds to, among other ion and G protein-coupled glutamate channels, the NMDA receptor. This results in calcium influx, which in turn results in the conversion of L-arginine to nitric oxide. Nitric oxide then diffuses retrogradely to the presynaptic neuron where it stimulates more release of glutamate. This process results in a fulminating feed-forward system, resulting in synaptic plasticity.

Pharmacological studies have shown that NMDA receptor antagonists (Bilsky et al., 1996; Elliott et al., 1994; Popik and Kozela, 1999; Trujillo and Akil, 1991) and nitric oxide synthase inhibitors (Babey et al., 1994; Elliott et al., 1995; Kolesnikov et al., 1992) prevent development of opioid-mediated tolerance (Drdla et al., 2009). Intense industrial initiatives to develop clinically useful NMDA receptor antagonists have largely been hindered by limiting motor and cognitive side effects (Salter, 2005). However, these side effects are thought to be attributable to non-selective or high affinity NMDA receptor antagonists (Wood, 2005). Low affinity (memantine, amantadine) or NR2B subunit-selective antagonists (ifenprodil) have recently been promoted as demonstrating a higher therapeutic index (Layton et al., 2006). These aforementioned adverse side effects are common limitations of traditional pharmacotherapeutic strategies; the proposed studies will circumvent these limitations by developing a gene therapeutic strategy augmenting an endogenous neurotransmitter thought to counter opioid tolerance.

The motor dysfunction of most NMDA receptor antagonists (i.e. MK801) can be detected on behavioral assays of motor dysfunction, such as rotarod. Interestingly, agmatine, a NMDA anatgonist, has no activity in the rotarod test (Fairbanks et. al., 2000).

It is noteworthy that such side effects are primarily observed with non-selective or high affinity NMDA receptor antagonists. It has been suggested that the NR2B subunit-selective antagonists have a lower side effect profile than traditional non-selective NMDA receptor antagonists due to site-restricted expression in the dorsal horn of the spinal cord (Mony, 2009). That restricted expression pattern predicts, and is consistent with, an expanded therapeutic window for NR2B selective antagonists (Suetake-Koga et al., 2006) relative to non-selective NMDA receptor antagonists. Based on observations that agmatine exhibits low toxicity, we have proposed that it may act selectively on the NR2B subunit of the NMDA receptor. The experiments to test that proposal are an ongoing line of investigation in Dr. Fairbanks's research program, but are not represented in this thesis research program.

1.2.3 *Decarboxylated Arginine (Agmatine): An NMDA Receptor Antagonist*

Agmatine, the decarboxylated form of L-arginine, was discovered in the CNS in 1994 and was subsequently shown to inhibit NMDA-evoked current (Yang and Reis, 1999) and behavior (Roberts et al., 2005) as well as NO production (Galea et al., 1996). Later studies of exogenously delivered agmatine showed an inhibitory role in multiple models of neuroplasticity (Piletz et al., 2013). Fairbanks and colleagues have demonstrated repeatedly that agmatine also prevents the development of opioid-induced tolerance (Fairbanks and Wilcox, 1997; Kitto and Fairbanks, 2006; Kolesnikov et al., 1996; Morgan et al., 2002; Wade et al., 2008), similar to other NMDA receptor antagonists and NOS inhibitors, but without the common side effect of motor ataxia

(Fairbanks et al., 2000; Nguyen et al., 2003). What separates agmatine from other NMDA receptor antagonists and NOS inhibitors is that it can be produced *in vivo*. The cloning of a synthetic (Morrissey et al., 1995) and degradative enzyme systems (Iyer et al., 2002) revealed a modulatable system that is also thought to be expressed in the CNS. Determining whether endogenously produced agmatine inhibits the development of opioid tolerance or chronic pain or other types of neural plasticity will provide important information as to the usefulness of targeting the agmatine metabolic pathway for therapeutic treatment.

1.3 *Gene Transfer Approaches for Treatment of Chronic Pain*

Alternative approaches to pharmacotherapy are being developed through the field of gene therapy with the expectation to modify viruses to provide permanent therapeutic and beneficial treatments for a variety of CNS disorders. Viral vector gene transfer utilizes the innate ability of viruses to enter the cell and deliver genetic material for long-term expression of biotherapeutic proteins. As effective treatments for chronic pain are limited, there are a number of investigators exploring gene therapy for treatment of chronic pain, with the goal of providing long-lasting, constitutive pain relief with little to no side effects.

Chronic pain is defined as pain that persists (after healing has occurred) longer than 12 weeks. Chronic pain is widely believed to be a disease, with known changes in the nerves that deteriorate with time (IOM, 2011). As the mechanisms underlying the development and persistence of chronic pain become better understood, new

opportunities for genetic interventions may emerge. To date, there have been several primary gene therapy approaches explored for treatment of pre-clinical models of chronic pain. Two primary approaches will be reviewed in the in the next few sections.

1.3.1 *Herpes Simplex Virus: Endogenous Opioids*

It is known that viruses can be modified in such a way as to provide therapeutic or beneficial treatments in gene therapy. The use of the herpes simplex virus (HSV) to deliver genes is one common example of this technique. Most studies have shown benefits of gene therapy in animal models and recently Phase I human clinical trials using HSV vectors for irretractable cancer pain (Kumar, Ruchi, James, and Chidiac, 2011). Specific to pain relief, previous gene therapy studies have utilized HSV to carrying coding for endogenous opioids such as beta-endorphin (Wolfe, 2007) or pre-proenkephalin (Glorioso, 2003; Yoshimura 2001; Westlund, 2009; Westlund and Vera-Portocarrero, 2012; Yang et al., 2008). Because HSV has a natural affinity to transfect neuronal cells and establish a latent infection in the dorsal root ganglion sensory neurons of the host, the HSV is an effective and convenient method for the delivery of gene therapy. The HSV vector can also cross the blood brain barrier and will persist in neurons and have been shown to be effective as brain tumor treatments (Latchman, 2001) and other various neurological disorders (Hester, Foust, Kaspar, and Kaspar, 2009).

The HSV gene sequences that cause an active infection are modified so that lytic infection does not manifest. Yet, a major concern lies in the fact that HSV vectors at higher titers have the possibility of reverting back to a wild-type phenotype (Fialho,

2010; Jamieson, 1995) and therefore could potentially reactivate a latent HSV infection; thereby risking HSV induced encephalitis or death. Interruption to the transcriptional silence may be induced by external factors such as stress, trauma or exposure to certain drugs, resulting in a reactivation of the lytic cycle (Blondeau, Aoki, and Glavin, 1993). This means the HSV gene therapies are often developed with a reduced concentration or period of transgene expression, which may limit HSV vectors for the use in chronic pain conditions (Gillet, Macadangdang, Fathke, Gottesman, and Kimchi-Sarfaty, 2009).

1.3.2 *Adeno-Associated Viral Vectors*

The adeno-associated virus (AAV) has risen to the forefront of gene therapy (Beutler and Reinhardt, 2009). This is in large part due to lack of pathogenicity and its variety of serotypes that enable specific cell-targeted entry (Gao, Vandenberghe and Wilson, 2005). The AAV is also nonpathogenic and is naturally replication defective. Several cell surface receptors have been identified that allow for AAV to be categorized into different serotypes (Mason et al., 2010). To date, twelve functional serotypes have been discovered and continue to be characterized (Miyake et al., 2012). Most research has focused on AAV serotypes 1 through 9, which have considerable differences at the capsid amino-acid sequence level (Daya and Berns, 2008). Because the viral capsid is the primary interface between the host and vector genome, it is believed that the capsid moderates specificity of transduction.

A study by Zincarelli et al., determined the tissue tropism of nine AAV serotypes (all vectors similarly prepared) via one route of delivery (2008). The results yielded three

categories of expression and two classes of kinetics (Zincarelli, Soltys, Rengo, and Rabinowitz, 2008). The unique characteristics and tissue specificity of each AAV serotype has made AAV gene therapy a hopeful leader in the translation of animal-based research to human clinical trials (Beutler and Reinhardt, 2009) and is one of the reasons why AAV is currently the fastest growing viral vector used for gene transfer research and clinical trials (Daya and Berns, 2008). Just in the past five years the use of AAV vectors in world wide clinical trials, has risen from 4.4% to 5.5% (Wiley, 2014).

1.3.3 *Viral Vector Gene Transfer with Arginine Decarboxylase (ADC)*

As stated earlier, attempts to manage opioid tolerance for those requiring chronic opioid analgesic therapy using NMDA receptor antagonists/NOS inhibitors have been significant but with limited effectiveness. The escalating reluctance to use opioid medications brings a challenge of identifying new approaches to provide effective pain management.

Previous gene therapy approaches have, for the most part, attempted overexpression of opioid neuropeptides. There has not yet been an emphasis on developing gene therapy as a complement to the use of opioid medications. Such an approach could prevent the dose-escalation that arises with chronic opioid. Previous findings would suggest that agmatine may be a good candidate for such a therapy. Since agmatine is produced endogenously, the opportunity arises to develop a method for enhancing expression of arginine decarboxylase (ADC) to create agmatine locally and constitutively via gene therapy.

Region-directed genetic modulation of the agmatine metabolic pathway at the spinal level could be a highly innovative approach for control of opioid tolerance or chronic pain without interfering with ongoing neuroplasticity required for normal brain functions, such as learning and memory. If this approach is established with proof-of-concept studies, other opportunities to regionally modify the agmatine system will likely emerge. The ability to alter negative neuroadaptation (such as opioid analgesic tolerance) without interfering with appropriate neuroadaptation (such as learning and memory) could significantly improve the therapeutic options of using opioids for treatment of chronic pain.

1.4 *Thesis Objectives*

It is my central hypothesis that increasing the amount of the synthetic enzyme of arginine decarboxylase (ADC) expressed in the CNS will increase levels of agmatine and prevent the development of opioid analgesic tolerance in a site-specific manner. Through this thesis work, we sought to determine whether intrathecal delivery of adeno-associated virus serotype 5 - human arginine decarboxylase (AAV5-hADC) would result in gene transfer of hADC to CNS sites, relevant to sensory system and opioid receptor signaling. I also sought to determine if subjects (mice) treated with AAV5-hADC would be able to prevent the development of analgesic tolerance to either morphine or the opioid neuropeptide endomorphin-2. Such a result would be analogous to what is observed with intrathecal delivery of agmatine. I also sought to determine whether spinal cords extracted from AAV5-hADC-treated subjects (mice) have a higher concentration of

agmatine, which would support the proposal that increased expression of arginine decarboxylase results in the production of elevated agmatine in the CNS. Finally, following the observation that intrathecal injection of AAV5-hADC results in gene transfer to the choroid plexus of the fourth ventricle, I also sought to further evaluate the suitability of the choroid plexus cells as a target tissue for therapeutic gene transfer.

1.4.1 *Thesis Preview*

In this thesis, I first report the following: The effects of AAV5-hADC gene transfer in two models of opioid-induced tolerance. Second, I present the quantification of agmatine and putrescine in tissues of animals treated with AAV5-hADC. Lastly, I describe the *in vitro* expression pattern of AAV-GFP treated choroid plexus cultures, in both primary and immortalized cell cultures. The data presented in this thesis provides proof-of-concept that intrathecal delivery of AAV5-hADC viral vector results in region specific expression of human arginine decarboxylase, and elevated concentration of agmatine in spinal cord relative to control. Additionally, I determined that the intrathecal delivery of AAV5-hADC results in gene transfer of hADC to the choroid plexus of the 4th ventricle, suggesting an additional, previously unconsidered target for gene transfer for therapeutic treatment of pain. The chapters are summarized below.

1.4.2 *Chapter 2*

In Chapter 2, the expression and distribution of hADC following AAV5-hADC intrathecal delivery in mice is presented together with the functional effects of AAV5-hADC expression on analgesic tolerance induced by chronic systemic delivery of morphine and acute intrathecal delivery of high dose endormophin-2. Additionally, a study of immunoneutralization of presumed elevated endogenous agmatine indirectly supports the proposal that the overexpressed hADC produces spinal agmatine.

1.4.3 *Chapter 3*

In Chapter 3, the challenges associated with bioanalytical detection of agmatine in CNS tissue are reviewed. Application of the recently introduced technology of heat stabilization is described as well as additional modifications to a previously established HPLC technique to improve detection of agmatine as well as separation from its primary metabolite, putrescine. Finally, analysis of heat-stabilized spinal cord extracted from AAV5-hADC-treated mice compared to saline-controls for agmatine content is presented. The data support the proposal that AAV5-hADC treatment results in elevated agmatine levels in spinal cord.

1.4.4 *Chapter 4*

The choroid plexus may be an advantageous target for the delivery of AAV gene therapy for neurological disorders. Delivering AAV gene transfer via intrathecal injection utilizes the CSF in the subarachnoid space to reach the apical side (CSF side) of the choroid plexus. In Chapter 4, gene transfer to primary choroid plexus cultures, Z310 cells (an immortalized choroid plexus cell line), as well as choroid plexus explants were used to characterize the expression pattern and time course of diverse AAV serotypes to transduce epithelial cells of the murine choroid plexus.

1.4.5 *Summary*

Through implementation of my thesis research I have accomplished several objectives. First, I evaluated the functional impact of spinal expression of the synthetic gene of agmatine: I found that overexpression of ADC resulted in a similar pharmacological profile as that observed when agmatine is given intrathecally (Fairbanks and Wilcox, 1997; Wade et al., 2009). Second, I identified a new approach to improve the detection of agmatine in CNS tissue, enabling the observation that AAV-hADC treatment elevates agmatine in spinal cord. Third, I have established a new line of investigation to characterize gene transfer to choroid plexus for consideration of the usefulness of targeting that tissue for chronic pain management.

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CHAPTER 2: *Adeno-associated virus mediated gene transfer of arginine decarboxylase to the central nervous system reduces morphine analgesic tolerance.*

CHAPTER SYNOPSIS

The decarboxylated form of L-arginine, agmatine, has been shown to prevent the development of opioid analgesic tolerance, dependence, and self-administration when given by both systemic and central routes of delivery. Agmatine has been reported to be located in brain and spinal cord, as has the expression of its synthetic enzyme, arginine decarboxylase (ADC). The presence of a biochemical synthesis pathway for agmatine offers the opportunity for overproduction of agmatine for local therapeutic delivery to regions where opioid analgesic tolerance is thought to arise. The effects of adeno-associated virus (AAV) gene transfer of human ADC (hADC) in two models of opioid-induced analgesic tolerance are reported here.

Eight weeks following initial intrathecal (i.t.) injections of vehicle- or AAV5-hADC-treated, mice were treated with repeated subcutaneous (s.c.) injections of either saline or morphine (s.c.) for 3 days. On day 4 morphine (s.c.) challenge dose-response curves were constructed in all treatment groups. In vehicle-treated subjects, a significant 4-fold rightward shift in the morphine dose-response curve was observed in the morphine treatment group relative to saline control, as expected. However, in subjects pre-treated with AAV5-hADC, the morphine dose-response curves between saline-pretreated and morphine-pretreated groups were equivalent, indicating that morphine analgesic tolerance did not develop. Similarly, AAV5-hADC pre-treatment prevented the development of acute endomorphin-2 analgesic tolerance, an effect reversed by intrathecal injection of

anti-agmatine, but not normal, IgG. Post-experiment, mRNA analysis confirmed that AAV5-hADC-treated mice demonstrated presence of hADC mRNA in periaqueductal grey (PAG), fourth ventricle choroid plexus, cervical and lumbar spinal cord as well as corresponding dorsal root ganglia. We conclude that the observed AAV5-hADC-mediated prevention in morphine tolerance is associated with the expression of hADC in regions relevant to the sensory system.

INTRODUCTION

A panel of complex side effects often accompanies long-term opioid pharmacotherapy, including, but not limited to, the development of opioid analgesic tolerance. The development of analgesic tolerance for patients on chronic opioid therapy can result in significant dose escalation (Chu et al., 2012; Duarte et al., 2012; Lorenz et al., 2002) which in turn result in an increase of adverse side effects (Chu et al., 2012), or a need for more invasive delivery approaches or the inability to adequately control pain ("ceiling effect") (Lorenz et al., 2002). Multiple strategies are used with varying success for such patients such as opioid rotation, drug holiday, or inclusion of adjuvant analgesics (Lorenz et al., 2002). It is recognized that the development of opioid analgesic tolerance is a highly adaptable event; such recognition emphasizes the challenge that has been the decades-long search to control the development of analgesic tolerance and suggests the value of a more targeted or site-specific strategy (Gintzler and Chakrabarti, 2008).

It has previously been shown that the decarboxylation product of L-arginine, agmatine, prevents the development of opioid analgesic tolerance when given either

systemically (Kolesnikov et al., 1996) or centrally (Fairbanks and Wilcox, 1997; Kitto and Fairbanks, 2006; Wade et al., 2009). Evidence suggests that agmatine likely antagonizes the NMDA receptor (Roberts et al., 2005a; Wade et al., 2009; Yang and Reis, 1999) or inhibits the nitric oxide synthase enzyme (Auguet et al., 1995; Demady et al., 2001; Galea et al., 1996; Roberts et al., 2005a) to exert this anti-tolerance action. Twenty years of pharmacological studies have consistently shown that antagonism of the NMDA receptor (Bilsky et al., 1996; Elliott et al., 1994; Popik and Kozela, 1999; Trujillo and Akil, 1991) or inhibition of the nitric oxide synthase (NOS) enzyme (Babey et al., 1994; Elliott et al., 1995; Kolesnikov et al., 1992) inhibits the development of opioid-induced analgesic tolerance.

Since agmatine is synthesized by arginine decarboxylase, which is expressed in the central nervous system (CNS), an opportunity arises to develop an agmatine-based gene therapy with site-directed expression within the CNS. Toward this end, we have developed an adeno-associated viral vector that carries the gene for arginine decarboxylase to determine whether overexpression of agmatine in tissues relevant to pain and opioid neuropharmacology can reduce the development of opioid analgesic tolerance in mice. An emerging body of literature suggests that such a gene therapy approach is likely to be clinically translatable with a long-term expression (Beutler and Reinhardt, 2009).

We have previously reported (Vulchanova et al., 2010) that through the delivery method of intrathecal injection via direct lumbar puncture and i.v. mannitol pretreatment, in conscious mice, we are able to achieve robust gene transfer to sensory neurons with

expression of a reporter gene in the dorsal root ganglia and at all levels of the spinal cord. We observed that in subjects injected with AAV5-GFP, but *not* pre-injected with i.v. mannitol, AAV5-GFP expression was not observed in spinal cord or brain parenchyma, but was detected in choroid plexus. Thus we hypothesized that selective delivery to the choroid plexus could be achieved via AAV5-mediated viral vectors.

Through using a similar, but modified approach, we are able to achieve AAV5-mediated gene transfer of the human arginine decarboxylase (hADC) enzyme in lumbar and cervical spinal cord as well as supraspinal locations. We hypothesized that overexpression of hADC along the sensory system would result in elevation of endogenous agmatine and reduce the development of opioid tolerance. To test this proposal, the development of chronic systemic morphine tolerance in AAV5-hADC-treated mice and vehicle-injected controls was compared.

In this chapter, I describe our experimental design and biological evaluation of the impact of intrathecal delivery of AAV5-hADC-GFP, a vector that contains the synthetic gene for human arginine decarboxylase (hADC). The results of this study indicate that endogenously produced agmatine enhanced from overexpression of hADC in the dorsal root ganglia and spinal cord prevent the development of opioid tolerance.

MATERIALS & METHODS

Animals

All experimental subjects were 20 to 25g adult male ICR-CD1 mice (Harlan, Madison, WI). All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Chemicals

Morphine sulfate was a gift from the National Institute on Drug Abuse. Endomorphin-2 (Endo-2) (YPFF) was synthesized by the University of Minnesota's Microchemical Facility, Minneapolis, MN.

AAV Vector and Packaging Gene Construct Development

The strategy for developing the AAV is described briefly here. Human ADC cDNA was cloned into the EcoRI and Xho1 sites of plasmid pAAV-IRES-hrGFP (Agilent Technologies) with assistance from the laboratory of Prof. R.S. McIvor (University of Minnesota). This viral construct also contained the gene for green fluorescence protein (GFP). The resulting plasmid pAAV-ADC contains the hADC expression cassette (CMV promoter-hADC cDNA-IRES-GFP and bovine growth hormone polyadenylation signal) flanked by AAV2 inverted terminal repeats (Figure 2.1A). This vector was packaged into AAV5 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). The vector titer was 8.24×10^{12} vector genomes/mL (vg/ml) for AAV5-hADC-GFP.

Gene Expression by 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 mM of each forward and reverse primer in a total of 20 ml per reaction. The sequences of the primers used are shown in Table 1. 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).

Antinociceptive Testing

Nociceptive responsiveness was tested using the warm water (52.5°C) immersion tail-flick assay (Janssen et al., 1963). Baseline measurements of tail-flick latencies were collected on all subjects (for a sample of n = 42 , mean = 3.32 s, and S.D. = 0.65 s). Each animal's baseline was used as its own control. The percent maximum possible effect (%MPE) was calculated accordingly: %MPE = (postdrug latency – predrug latency)/(cutoff – predrug latency) x 100%. To avoid tissue injury, a maximum score of 100% was assigned to those animals not responding before the 12 second cutoff.

Statistics

In Table 3, the ED50 values and 95% confidence intervals (CIs) of drugs were calculated by parametric linear regression analysis of the log dose–response curves. A minimum of three to four doses was used to construct the dose–response curve for each pre-treatment group. When evaluating the significance of a potency shift between the experimental pre-treatment groups and the saline control group, a relative potency ratio was calculated and t-test ($p<0.05$) performed. These methods have been previously described by Tallarida and Murray (1987). These calculations were performed using the pharmacological statistics software FlashCalc (Dr. Michael Ossipov, University of Arizona, Tucson, AZ)

Injections

The AAV5-hADC construct was delivered intrathecally (i.t.) by direct lumbar puncture in awake mice (Hylden and Wilcox, 1980) by an experimenter (KFK) with over 20 years experience in this method of drug delivery. A minor modification of the protocol

was required to conserve the AAV5-hADC vector. The needle (30-gauge, 0.5-inch) was connected to a length of PE10 tubing, which was then connected to a second needle (a 30-gauge, 0.5-inch) that was attached to a 50-µl Luer-hub Hamilton syringe. For both AAV5-hADC experiments, 10 µL of the construct containing 8.24 $x10^{12}$ viral vector genomes were injected intrathecally. The injection was administered by gripping gently the iliac crest of the rodent and inserting the needle (bevel side up) at about a 45° angle centered on the ileac crest. A reflexive flick of the tail indicated puncture of the dura. Following the injection, the animals were returned to the vivarium where they remained until the day of rotarod assay, tolerance injections, and tail-flick assay. This was eight-weeks for the chronic morphine experiments and nine-weeks for acute morphine. Extraction of spinal cord, fourth ventricle choroid plexus, midbrain (PAG), liver and DRG for RT-PCR took place on the last day of behavioral testing, after all behavioral testing had been completed.

Chronic Opioid Analgesic Tolerance Induction: Morphine

Mice were made tolerant to the analgesic effect of morphine by repeated administration of subcutaneous morphine over the course of three days. A single injection of was delivered on day 1 (3 mg/kg). Three injections were delivered at 9 am, 5 pm, and 11 pm on Days 2 (3 mg/kg) and 3 (5 mg/kg). A total of seven subcutaneous injections were received over the course of the induction schedule. A control group of mice received an equivalent number of subcutaneous saline injections concurrently with the morphine-treatment group. All injections were delivered in a volume of 100 microliters/10 grams.

Acute Opioid Analgesic Tolerance Induction: Endomorphin-2

Mice were made tolerant to the analgesic effect of endomorphin-2 (Endo-2) by a single intrathecal injection of a large dose (10 nmol) of Endo-2. Baseline tail flick latencies were collected on all mice (52.5°C). Saline or Endo-2 was injected. Thirty minutes later, tail flick latencies were taken again and the latencies had returned to baseline levels. Cumulative dose-response curves were constructed in the following way. A single dose of Endo-2 was injected, and the tail flick latency was taken at 2.5 minutes later (the peak analgesic time point). Thirty-minutes later, a second cumulative dose of Endo-2 was delivered and the process repeated. Doses were delivered in this fashion until a complete dose-response curve was constructed.

Immunoneutralization Experiments: Opioid Analgesic AcuteEndomorphin-2 Tolerance

These experiments were conducted with the same cohort of animals used for the acute Endo-2 tolerance experiments. However, five minutes prior to the induction of acute Endo-2 tolerance, either normal guinea pig IgG (150 ng) or anti-agmatine IgG (150 ng) were injected intrathecally as a "pre-pre-treatment". These products were raised in-house and have been previously characterized by Wade and colleagues (2009). Cumulative Endo-2 dose-response curves were collected 30 minutes after the Endo-2 pre-treatments as previously described.

Rotarod Assay

After two training sessions, mice were given the opportunity to walk for a maximum of 300 s on an accelerating (4–40 rpm) rotarod (Ugo Basile, Varese, Italy). We compared the latency to fall between the four treatment groups.

RESULTS

Expression of Arginine Decarboxylase in the Sensory System

We hypothesized that overexpression of the synthetic enzyme for agmatine, arginine decarboxylase (ADC) in the sensory neurons of the dorsal root ganglia and spinal cord, would reduce opioid analgesic tolerance. By administering the vector described above (Figure 2.1A) we intended to confirm successful transduction of the viral vector in tissue, by green fluorescent protein (GFP) immunoreactivity. Unfortunately, in this case, the vector did not translate the GFP protein (Schuster, Schnell, and Fairbanks, data not shown). Therefore an alternative method was used to confirm successful vector transduction via RT-PCR for presence of hADC mRNA.

Rodents express a form of ADC that is distinct from that of the human sequence, since a human form of ADC was inserted, we were able distinguish between the two ADC forms (mouse and human) in transduced tissue. Thus enabling the identification of the efficacy of gene transfer to the spinal cord and dorsal root ganglia. Our laboratory team members developed specific primers that could distinguish detection of human ADC (Table 1) from that of mouse ADC in order to be able to assess the outcome of gene transfer (Schnell, Fairbanks, et al., unpublished observation). In Figure 2.1B, it was shown that these specific primers can detect native human ADC present in human embryonic kidney (HEK) 293 cells, but do not detect mouse ADC in spinal cord tissue extracted from mice treated with AAV5-GFP, a standard control used in *in vivo* experiments. These primers also fail to detect mouse ADC in mouse fibroblast 3T3 cells, but detect human ADC in 3T3 cells infected with AAV5-ADC-GFP vector. These experiments confirm the specificity of these primers for the human form of ADC.

In two separate sets of subjects, 8.24 x 10^{10} vector genomes of AAV5-hADC-GFP were injected intrathecally by direct lumbar puncture into nerve-injured mice. The expression of hADC was evaluated along the neuraxis at lumbar and cervical levels both in spinal cord and DRG. In subjects treated with AAV5-hADC, mRNA for hADC was detected in at least one tissue in 94% of the subjects (Table 2). The hADC transcripts were not detected in any tissue in saline-treated subjects. RT-PCR analysis on anatomic components of sensory system-related tissues (e.g. lumbar and cervical spinal cord and DRG) revealed distribution along the neuraxis of the spinal cord and distally into portions of the brain (Figure 2.1C). This tissue distribution and the percentages of AAV5-hADC-GFP expressed in these tissues are summarized in Table 2.

AAV5-hADC Expression on Opioid Analgesic Tolerance

Having established that the AAV5-hADC construct transduced tissues relevant to the sensory pathway, as well as in regions that express the mu opioid receptor, our goal was to characterize the dose-response relationship of morphine in the condition of chronic systemic morphine tolerance in normal mice and in mice with elevated hADC expression. Therefore, dose-response curves were determined for the antinociceptive effects of morphine in the tail flick test in mice that had been intrathecally injected eight weeks prior with either AAV5-hADC or saline as a control to the gene therapy treatment. These two separate cohorts were then further divided into two groups; which received a series of seven subcutaneous saline or morphine injections over the course of three days. On day 4 all subject groups were subcutaneously injected sequentially with increasing

doses of acute challenge morphine (1, 3, 10 mg/kg.) mice. Acute challenge morphine dose-response curves were then constructed and compared between the saline and morphine-injected groups for each cohort (AAV5-hADC and saline eight week pretreatment groups). This general experimental design for this chronic morphine tolerance study in AAV5-hADC treated animals is illustrated in Figure 2.2.

The outcomes were then compared between the two cohorts. In the intrathecal saline pre-treatment group, repeated injections of s.c. morphine produced a 4-fold rightward shift in the morphine dose-response curve (Figure 2.3A) relative to the morphine dose-response curve in the group that received repeated s.c. saline (control group). Repeated morphine injections increased the acute morphine ED50 value (Table 3). The significant rightward shift confirms the induction of systemic morphine tolerance in this model in the control condition. In contrast, when mice were pre-treated with intrathecal AAV5-hADC, repeated injections with morphine did not produce a significant shift in the acute challenge morphine dose-response curve compared to those repeatedly injected with s.c. saline (Figure 2.3B). The ED50 values for morphine were equivalent between the two repeated injection groups (Table 2.3). The observation of no shift in the morphine dose-response curves indicates that morphine tolerance did not develop in subjects receiving the AAV5-hADC injection i.t..

Anti-Agmatine IgG Reverses AAV5-hADC Inhibition of Opioid Analgesic Tolerance

To further determine whether the prevention of opioid tolerance in AAV5-hADC treated mice was attributable to an effect of the agmatine molecule, an alternative indirect

approach was used: Immunoneutralization. The impact of intrathecal delivery of an immunoneutralizing antibody (IgG-Ag) (Wade et al., 2009) was assessed in a model of opioid induced analgesic tolerance. For this experiment, the opioid neuropeptide, endomorphin-2 (Endo-2) (YPFF) (Zadina et al., 1997) was used. It was previously demonstrated (Stone 1997; Wade 2009) that higher intrathecal doses of Endo-2 (10-30 nmol, it.) develop acutely induced analgesic tolerance. It was also shown previously, that intrathecal pre-treatment of an immunogammaglobulin (IgG) raised to selectively target agmatine (Fairbanks, 2000; Wade, 2009) sensitizes mice to the development of Endo-2 analgesic tolerance induced by low doses (Wade 2009). We elected to use this model of acute opioid tolerance to test for the effect of immunoneutralization of agmatine in the hADC-treated mice. See Figure 2.4 for the experimental design.

In this experiment, a cohort of age-matched mice served as controls to the AAV-hADC treated mice. This control cohort was injected with either intrathecal saline or high dose of Endo-2 (10 nmol) to establish the magnitude of acute endomorphin-2 analgesic tolerance. Thirty minutes after the initial high dose injection, mice were tested cumulatively with increasing doses of Endo-2. The tail flick test was performed 2.5 minutes after the test injection. In this experiment, control mice pre-treated with saline demonstrated analgesic potency with an ED50 value of 7.3 nmol. Control mice pre-treated with Endo-2 mice did not display analgesic efficacy and, therefore, acute tolerance was induced (Figure 2.5). In contrast AAV5-hADC-treated mice that were pre-treated with Endo-2 demonstrated Endo-2 analgesia with a relative potency to saline of 1.9 nmol, comparable to that of control mice pre-treated with saline (Figure 2.6). These

same AAV5-hADC treated mice had received a pre-pre-treatment of normal GP-IgG (150 ng) as control to the AAV5-hADC treated mice that received a pre-pre treatment of anti-agmatine IgG (150 ng) prior to the high dose of Endo-2 used to induce acute tolerance. The analgesic efficacy of Endo-2 in those mice (AAV5-hADC, Anti-Ag-IgG, Endo-2) was significantly ablated, comparable to those control mice that received a pre-treatment of Endo-2 and in which acute tolerance to Endo-2 was demonstrated (Figure 2.6). This last experimental group shows that when the anti-agmatine IgG is given as a pre-pre-treatment to Endo-2 in the AAV5-hADC mice, that the protective effect of the AAV5-hADC treatment is reversed. We interpret this to mean that the elevated concentrations of agmatine are bound by the anti-agmatine IgG, rendering the elevated agmatine unavailable to exert its anti-opioid tolerance effect. This experiment supports the proposal that overexpression of AAV5-hADC results in production of agmatine that acts to prevent the development of spinal opioid analgesic tolerance induced by Endo-2 and, perhaps, analogously morphine.

Motor Impairment Assay

In other experiments it has been observed that repeated intrathecal delivery of the primary metabolite of agmatine, putrescine, results in motor impairment (Peterson and Fairbanks, unpublished observations). Such an observation raises a concern about the constitutive production of agmatine from overexpression of hADC. To assess this potential toxicity, we evaluated the motor performance of all animals by the rotarod assay, a test frequently used to assess motor dysfunction. Prior to the morphine tolerance

experiments, all subjects were assessed for motor impairment by evaluating their performance in the accelerating rotarod assay for mice (Fairbanks et al., 2000). Following two training sessions, mice are allowed to walk for 300 s on a accelerating rotating rod. Normal mice can readily achieve this goal whereas mice with motor impairment are not able to complete the session. No differences in rotarod performance were observed between treatment groups (Table 4). These data indicate that the hADC-treated mice displayed no evidence of motor toxicity under the conditions of our experiments.

Taken together, these data suggest that expression of human ADC in the central nervous system of mice treated with AAV5-hADC prevents the development opioid analgesic tolerance.

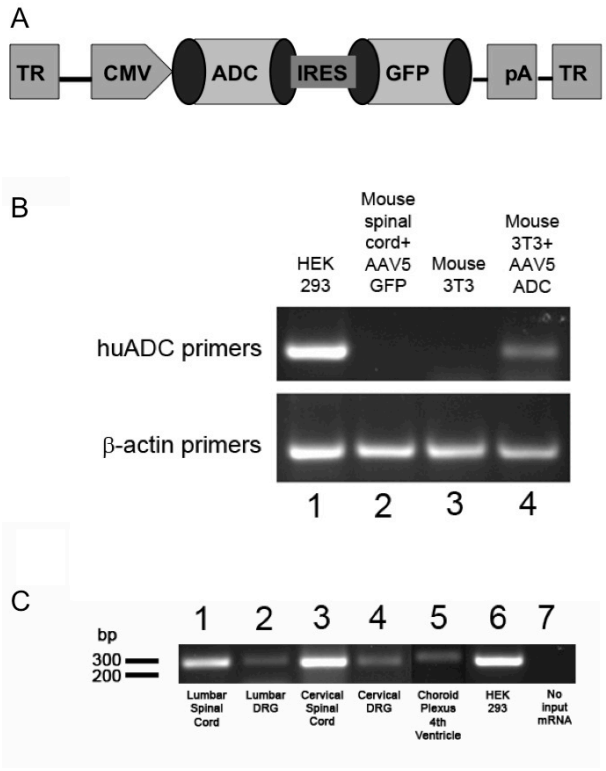

Figure 2.1: Intrathecal delivery of AAV5-ADC into adult mice results in widespread AAV5-ADC transduction associated with high-level expression of ADC throughout the spinal cord, dorsal root ganglia and 4th ventricle choroid plexus. **(A)** Depiction of the therapeutic AAV construct. the human hADC cDNA regulated by a CMV promoter. This construct was packaged using AAV serotype 5 capsid or AAV serotype 9 capsid to produce AAV5-ADC or AAV9-ADC. GFP was included in the construct separated from human ADC by an internal ribosome entry site (IRES) nucleotide sequence. **(B)** All PCR products were specific for each target. Human embryonic kidney cells (HEK293) were used as a positive control for hADC primers (lane 1, 273bp product); AAV5-GFP injected mouse spinal cord and wild type 3T3 cells were used as negative controls for hADC primers (lanes 2 and 3). No hADC mRNA was detected in mouse tissue or 3T3 fibroblast cell lines unless it had been infected with the AAV5-hADC construct; beta actin mRNA (250 bp product) was detected in all samples (lanes 5-8). **(C)** After infection by intrathecal injection of the AAV5-hADC construct, hADC mRNA was detected near the site of injection (lumbar spinal cord and DRG) as well as further rostrally (cervical spinal cord and DRG) and in non-neuronal populations of the choroid plexus. No PCR products were observed when either no RNA was used (no input mRNA) or no reverse transcriptase was included in the reverse transcription step (not shown) (Schnell and Fairbanks, unpublished observations).

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

Table 2.1: Primer sequences used in RT-PCR experiments (Schnell and Fairbanks, unpublished data).

Tissue Region	Periaqueductal Gray (PAG)	Choroid Plexus (4th Ventricle)	Cervical DRG	Cervical Spinal Cord	Lumbar DRG	Lumbar Spinal Cord	hADC Expression in Any Tissue
Day 60 Post AAV5-hADC Injection (n=16)	50% (8/16)	87% (13/15)	75% (12/16)	88% (14/16)	81% (13/16)	88% (14/16)	94% (15/16)

Table 2.2: Percentage of AAV5-hADC-GFP Transduction. The hADC mRNA expression in various CNS tissue (DRG) at t=0 days following the initial intrathecal injection. Periaqueductal gray, choroid plexus, spinal cord (lumbar, cervical), and DRG (lumbar, cervical) 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.

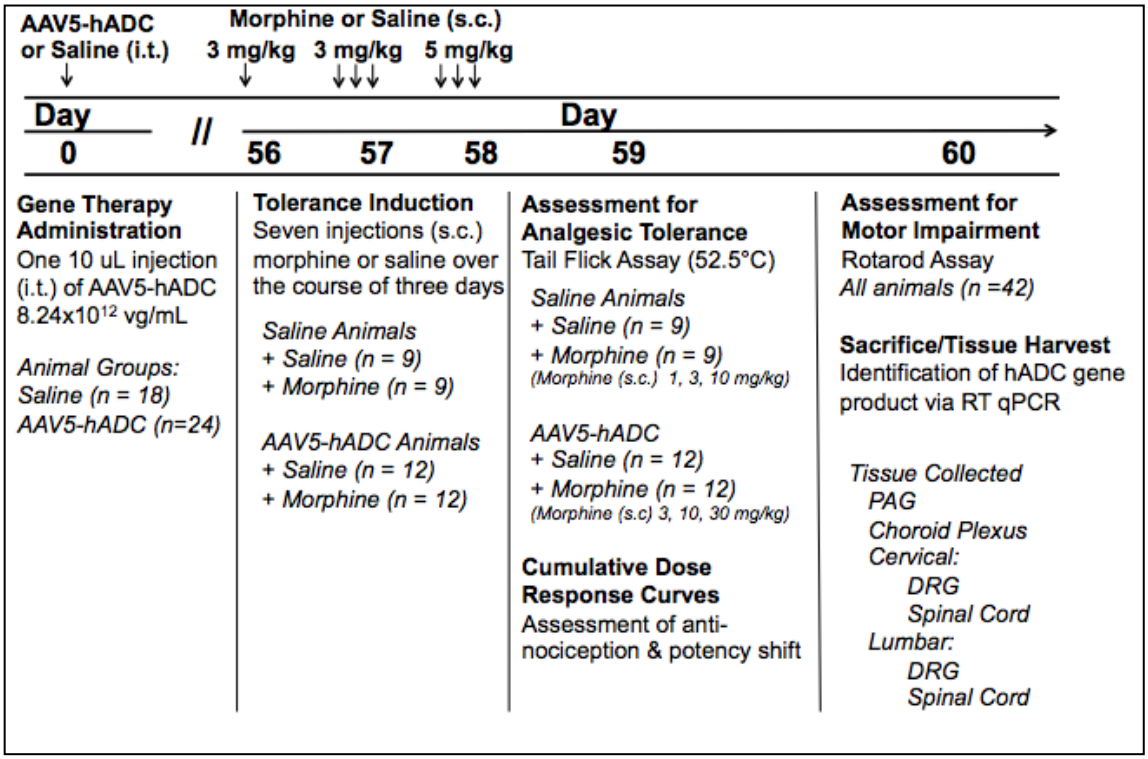

Figure 2.2: Study Timeline: Morphine Tolerance in animals treated with AAV5-hADC.

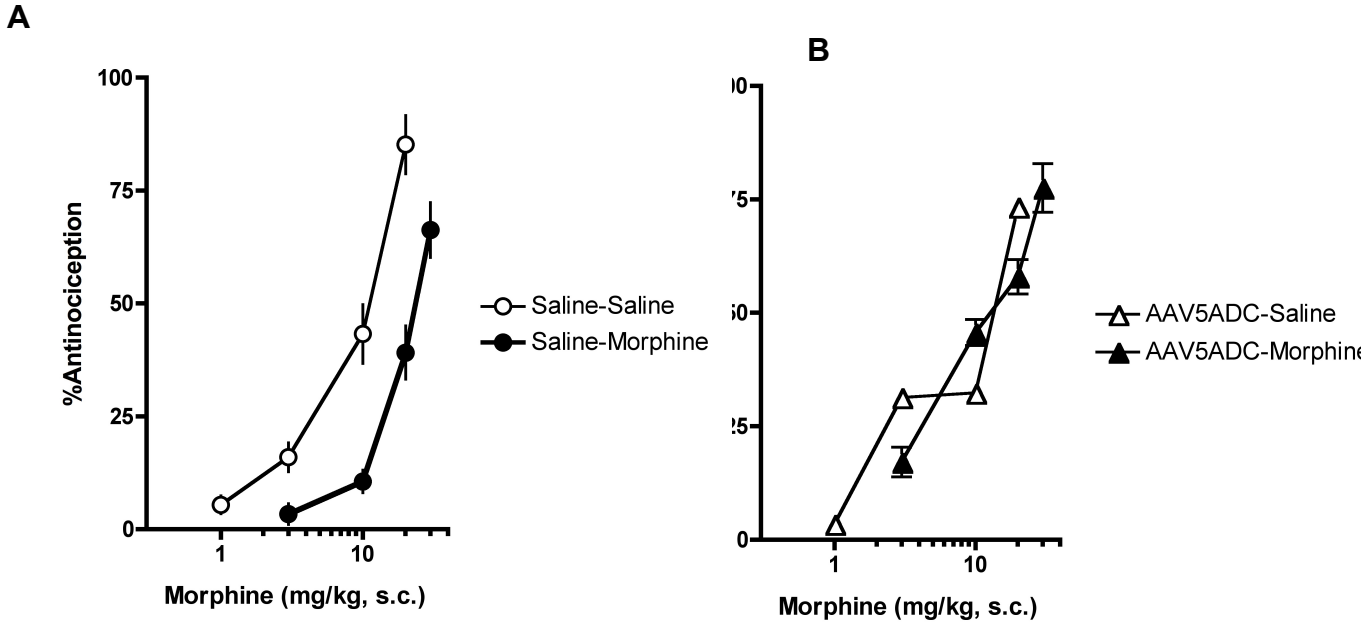

Figure 2.3: Effects on AAV5-hADC pre-treatment development of morphine tolerance. Analgesic dose-response curves to acute subcutaneous morphine challenge on Day 4 following repeated subcutaneous injections of either saline or morphine **(A)** *Control subjects*. Repeated s.c. injections with morphine (closed circles) decreased acute morphine potency and efficacy compared with subjects repeatedly injected with saline (open circles), indicating induction of chronic morphine analgesic tolerance. (**B)** *AAV5-hADC treated subjects*. Repeated s.c. injections with morphine (closed triangles) demonstrated equivalent acute morphine potency and efficacy compared with subjects repeatedly injected with saline (open triangles), indicating that induction of chronic morphine analgesic did not occur in this experimental group.

Group	ED50 Value (mg/kg, s.c.)	Relative Potency Compared to Saline
Saline - Saline	7.2 (5.7 – 9.1)	1
Saline - Morphine	27.8 (20.3 – 38.0)*	3.9
AAV5-hADC – Saline	10.4 (7.8 – 13.9)	1.4
AAV5-hADC - Morphine	11.9 (10.0 – 14.0)	1.65

Table 2.3: ED50 values and relative potency for all treatment groups.
(* Significant difference relative to the saline pretreatment group)

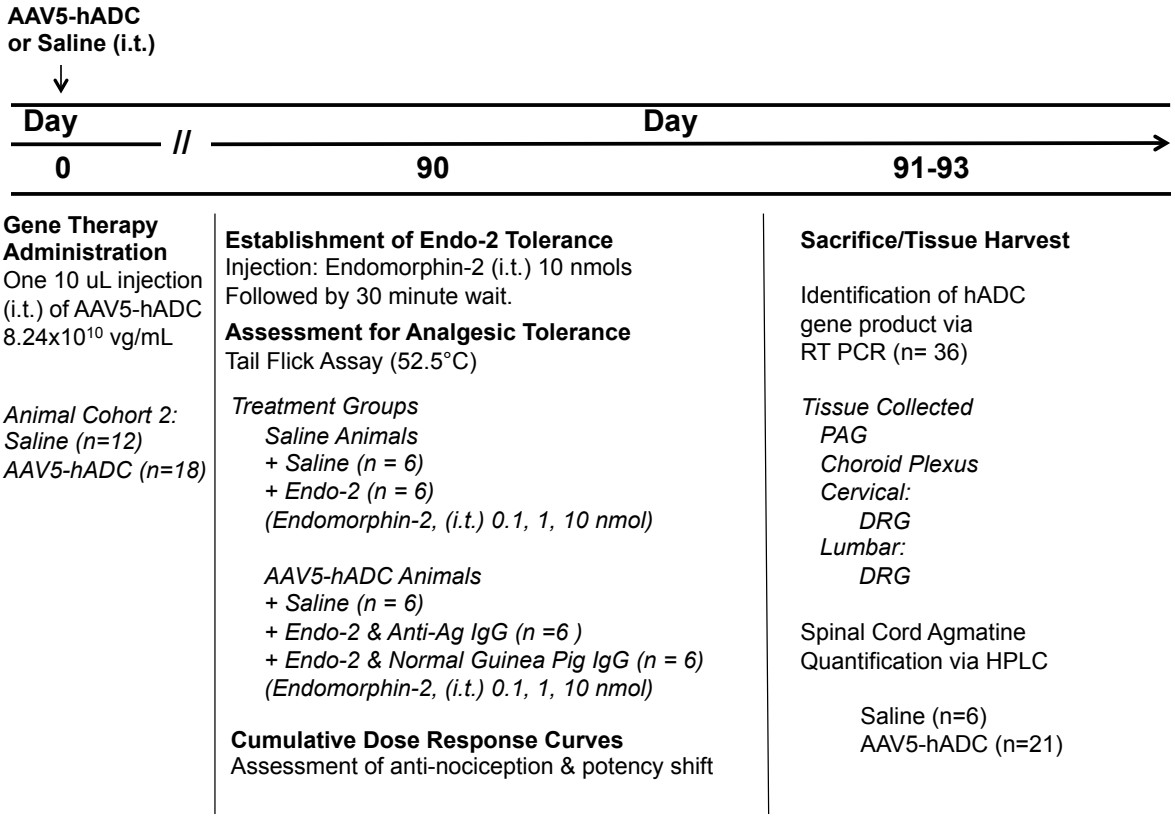

Figure 2.4: Study Timeline: Acute endomorphin-2 Tolerance in AAV5-hADC-treated animals.

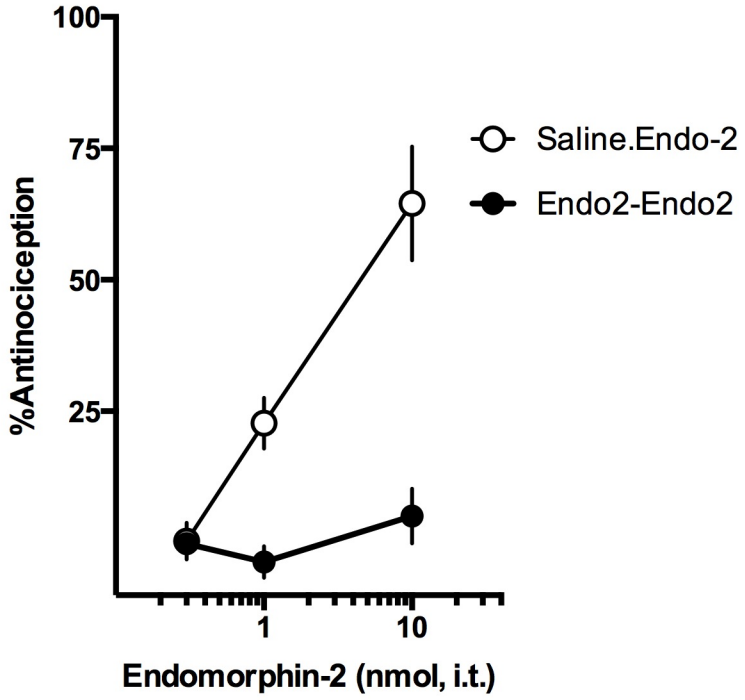

Figure 2.5: Endomorphin-2 Acute Tolerance. Analgesic dose-response curves to acute intrathecal endomorphin-2 challenge 30 minutes following an acute injection of either saline or endomorphin-2. A high dose of endo-2 (10 nmol) reduced endo-2 analgesic efficacy (closed circles) compared with subjects injected with saline (open circles), indicating induction of endomorphin-2 analgesic tolerance. Saline-Pretreatment (white circles) ED50= 7.3 nmol (95% CI = 3.6 – 14.7 nmol).

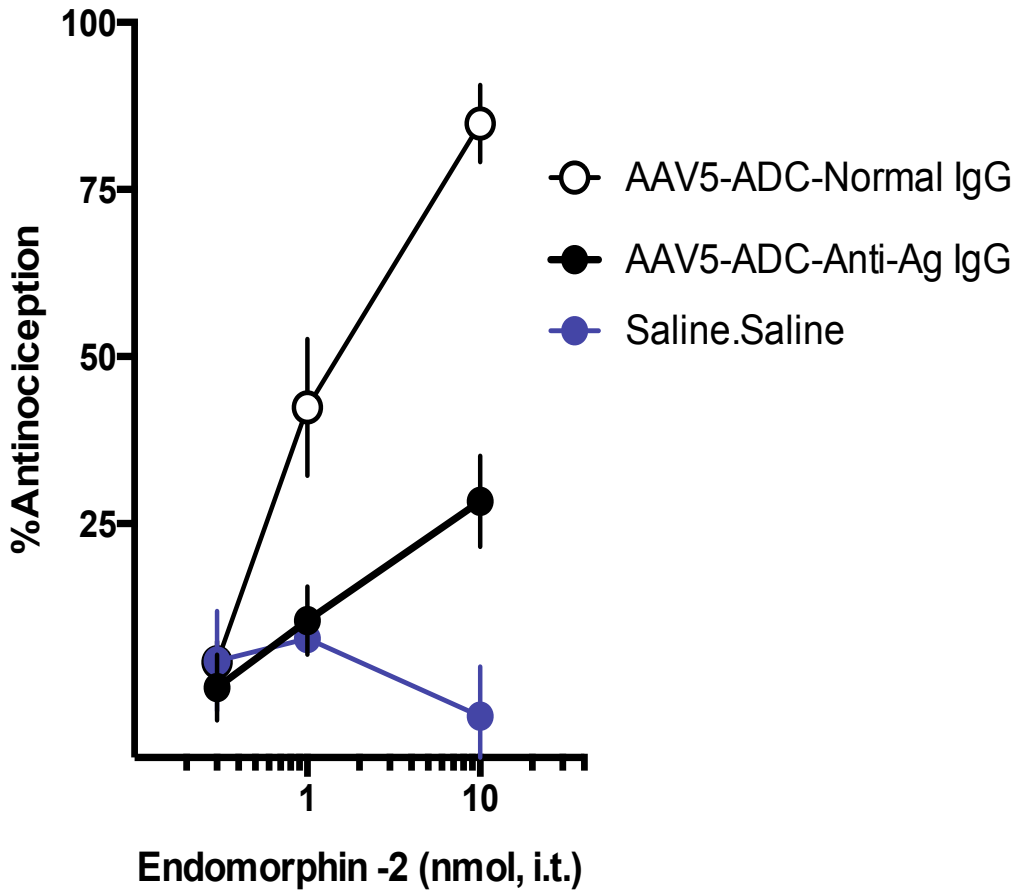

Figure 2.6: Effects on AAV5-hADC pre-treatment development of endomorphin-2 (Endo-2) tolerance in subjects pre-treated with normal IgG or anti-agmatine IgG.
Analgesic dose-response curves to acute intrathecal Endo-2 challenge; 30 min following an acute intrathecal injections series of either Normal IgG + Endo-2 (open circles) or anti-agmatine IgG + Endo-2 (closed circles). A group of age-matched controls (blue circles) injected with saline paired to those injected with AAV5-ADC were injected saline and then endomorphin-2. Saline + Saline resulted in decreased acute Endo-2 efficacy (blue circles). AAV5-hAD + Normal IgG did not result in development of analgesic tolerance to Endo-2 (Open circles). AAV5-hADC + Anti-Ag IgG treated subjects pre-treated with Endo-2 demonstrated reduced Endo-2 efficacy indicating that induction of acute Endo-2 analgesic tolerance did occur in this experimental group. AAV5-hADC+Normal IgG-Endo-2 (white circles) ED50= 1.9 nmol (95% CI = 1.3 - 2.8 nmol).

Treatment Group	Average Time on Rotarod (seconds)
Saline - Saline	288 (270-305)
Saline - Morphine	285 (265-304)
AAV5-hADC – Saline	270 (243-296)
AAV5-hADC - Morphine	278 (243-313)

Table 2.4: Rotarod Motor Dysfunction: Comparison between treatment groups on performance in the rotarod assay of motor impairment.

DISCUSSION

The key observation in the present study is that intrathecal delivery of a viral vector carrying the gene for human ADC resulted in the prevention of the development of opioid analgesic tolerance in mice eight weeks post-injection. Expression of mRNA coding for the human ADC gene was found in cervical and lumbar spinal cord and dorsal root ganglia, as well as fourth ventricle choroid plexus, and periaqueductal grey (PAG). These results indicate that regions of the CNS that are known to participate in the development of opioid analgesic tolerance carried the target mRNA. Therefore, it is feasible that agmatine was produced by the overexpressed enzyme resulting in outcomes that parallel the pharmacological studies of exogenous agmatine delivery to the intrathecal space.

In order to pursue the identification of endogenous agmatine arising from the AAV-hADC treatments, we took an initial indirect approach. An immunoneutralization strategy used scavenging antisera to determine whether endogenous agmatine accounted for the prevention of opioid tolerance observed in subjects treated with the AAV-hADC treatment. This method has been widely used to assess the effects of endogenous analgesic substances (Ohsawa et al., 2001; Tseng et al., 2000; Vanderah et al., 1994) and recently endogenous pro-nociceptive substances (Fairbanks et al., 2014). It was previously demonstrated that a structure-specific anti-agmatine immunoglobulin G (Anti-Ag IgG) reversed the pharmacological effects of exogenously applied intrathecal agmatine (Wade et al., 2009). Therefore, it was reasoned that intrathecal administration of the Anti-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 analgesic tolerance was significantly reduced. The selectivity of this effect for agmatine is indirectly supported by the observations that such a reduction was not observed with and intrathecal injection of normal IgG. Specifically, the lack of effect of the normal IgG indicates that Anti-Ag IgG reversal of the effect of AAV-hADC overexpression on analgesic tolerance is not an incidental effect of the normal IgG. Therefore, these observations support the proposal that elevated endogenous agmatine contributes to the anti-tolerance effect of the AAV5-ADC treatment.

Agmatine, was discovered in the CNS in 1994, closely followed by the cloning of its synthetic (Morrissey et al., 1995) and degradative enzyme systems (Iyer et al., 2002) that are also expressed in CNS. Subsequently neuropharmacological investigations have converged on an inhibitory role in multiple models of behavioral neural plasticity (Piletz et al., 2013). Specifically, we and others have shown that agmatine also robustly inhibits the development of opioid-induced tolerance (Fairbanks and Wilcox, 1997; Kitto and Fairbanks, 2006; Kolesnikov et al., 1996; Morgan et al., 2002; Wade et al., 2008), but without motor toxicity (Fairbanks et al., 2000; Nguyen, 2003). Consistently, in the present study, subjects receiving the i.t. injection of AAV5-hADC did not demonstrate altered performance in rotarod compared to control subjects at the time point assessed.

It has previously been shown that agmatine inhibits NMDA-evoked current (Yang and Reis, 1999) and behavior (Roberts et al., 2005) as well as NO production (Galea et al., 1996). The collective prior indirect evidence suggests that agmatine likely acts

through antagonism of the NMDA receptor/NOS cascade. It has been established for over two decades that co-administration of NMDA receptor antagonists with opioids prevents the development of opioid tolerance (Trujillo and Akil, 1992). This strategy has been used clinically to "reset" a patient's opioid analgesic efficacy and potency when opioid analgesic tolerance has arisen (Prommer, 2012; Quinlan, 2012). However, initial efforts to develop a combined preparation of opioid + NMDA receptor antagonist have not yet advanced to practice (Galer et al., 2005). As stated previously, what distinguishes agmatine from other NMDA receptor antagonists/NOS inhibitors is that it is synthetized in vivo. Therefore, through a gene therapy approach such as described here, highly localized delivery at the site of maladaptive neuroplasticity becomes a possibility.

Prior studies of the distribution of GFP labeling in spinal cord and DRG (Vulchanova et al., 2010) suggest that the AAV5 serotype used in these experiments differentially target sensory neurons. Specifically, there is preferential tropism for large diameter sensory neurons and small diameter CGRP- or SP-containing neurons. Additionally, we have demonstrated expression of GFP in various regions of the brain (Schuster et al., 2014), including the choroid plexus, presumably from rostral diffusion of the viral particles. Therefore, the mRNA expression data presented here are consistent with these prior gene expression distribution studies using the GFP marker.

The tissues assessed for hADC expression merit speculation as to how they may participate in the observed prevention of opioid tolerance. It is conceivable that if hADC is overexpressed in sensory neurons terminating in the dorsal horn of the spinal cord, or in intrinsic spinal neurons, the resultant elevation in agmatine production could act

similarly to that of exogenously injected agmatine in models of morphine tolerance (Fairbanks and Wilcox, 1997). Additionally, the expression of hADC in epithelial cells of the choroid plexus could be participating in the elevation of agmatine via release of agmatine into the CSF. This could cause a broad distribution of the molecule throughout the CNS that could act in a comparable manner as observed in prior pharmacological studies demonstrating efficacy of i.c.v. injected agmatine on morphine analgesic tolerance (Kitto and Fairbanks, 2006). In regards to the expression of hADC in the PAG, to our knowledge, there has not yet been an evaluation of agmatine microinjected into the PAG. It is also possible that hADC expressed in PAG could produce agmatine that could modify morphine antinociception. However, the PAG is less likely to be a locus for prevention of the development of opioid analgesic tolerance in light of previous evidence that microinjections of MK801 to the PAG does not impact development of morphine analgesic tolerance (Morgan et al., 2009). We cannot at this time determine whether expression in one particular region or another is primarily responsible for the effect of the treatment. Further work is needed to selectively transduce one region or one cell population to determine where the effect of hADC expression originates. Additionally, hADC expression may have taken place in a location that we have not probed that may also contribute to the observed effect.

SUMMARY & CONCLUSIONS

The present study features an application of gene transfer to sensory neurons, the midbrain, and choroid plexus epithelial cells using direct lumbar puncture. As gene therapeutics are developed, optimized, and considered for translation into clinical practice for applications (Aubourg, 2013), an opportunity to intrathecally deliver gene therapy as an adjuvant to chronic opioid pharmacotherapy may be an effective pain management strategy.

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Chapter 3. *Quantification of agmatine in mice intrathecally injected with AAV5-hADC.*

CHAPTER SYNOPSIS

The data presented in Chapter 2 strongly support the proposal that the expression of human ADC following intrathecal delivery of viral vectors results in elevation of agmatine in spinal cord. In order to confirm that proposal it is necessary to determine whether tissue extracted from hADC-treated subjects contain elevated agmatine levels relative to controls. The goal of this study was to optimize the existing HPLC quantification method of agmatine in order to enable such analysis of hADC-treated tissue. Until recently, detection of agmatine in mouse spinal cord has largely been elusive and confounded by excess levels of the metabolite of agmatine, putrescine. Based on the introduction of heat stabilization technology, and minor solvent modifications of the previously validated method by Roberts et al., (2007) we hypothesized that application of such an approach would improve detection of agmatine in mouse spinal cord. The impact of heat stabilization of tissue samples versus snap-frozen (liquid nitrogen) was evaluated in terms of both agmatine and putrescine detection (Figure 3.4). The final modified method described here was used to evaluate levels of agmatine in spinal cord tissue of mice receiving a single i.t. injection of AAV5-hADC.

INTRODUCTION

Agmatine, (4-(Aminobutyl)guanidine) is a small molecule that is synthesized from L-arginine by arginine decarboxylase (ADC) (Figure 3.1). First discovered in 1910 by the Nobel laureate, Albrecht Koessel, in the pollen of *Ambrosia artemisfolia*, agmatine was primarily thought to be a constituent of bacterial and plant organisms. However, it was not until 1994 that agmatine was identified as an endogenous substance in the central nervous system by Donald Reis and colleagues at Cornell Medical college (Li et al., 1994). Since then it has been demonstrated to have numerous properties that are suggestive of a neurotransmitter. Notably, agmatine appears to have an inhibitory impact on neuroplasticity (Piletz et al., 2013). These observations would suggest that agmatine is both measurable and manipulatable in the CNS. The present chapter focuses on a new strategy that overcomes challenges that we have observed in measuring picomol levels of agmatine in CNS tissues. The modifications to the previously validated method by J. Roberts (2007), improves detection level of agmatine and reduces the opportunity for putrescine, the metabolite of agmatine, to interfer with the stoichiometry of the derivatization reaction (Figure 3.2B).

The current understanding of the *de novo* synthesis of agmatine and putrescine in mammals is that L-arginine is converted to agmatine via arginine decarboxylase (EC 4.1.1.19) (Li et al., 1994; Regunathan and Reis, 2000). Agmatine is subsequently hydrolyzed by the enzyme agmatinase (EC 3.5.3.11) into putrescine (Figure 3.1) (Gilad et al., 1996; Sastre et al., 1996). It has been noted that human ADC is not the same as the ADC found in plants, bacteria or invertebrates (Molderings and Haenisch, 2012). A

partial clone of the human form of ADC was introduced in 1995 by Morrissey and colleagues (Morrissey et al., 1995). The human form of the putrescine enzyme, agmatinase, was cloned in 2002 (Iyer et al., 2002; Mistry et al., 2002). These enzymes have been shown to be differentially distributed in murine neuronal cell bodies (Zhu et al., 2004), glial cells hippocampus and cerebral cortex (Peters et al., 2013; Iyo, 2006).

Agmatine Concentrations in Central Nervous System

The first studies of the tissue distribution of endogenous agmatine by Raasch et al, (1995) observed a concentration in extracted Sprague Dawley rat whole brain of 2.4 ng/g ww (18 pmol/g) (Table 3.1). Lung and stomach tissue respectively demonstrated 10- and 50-fold greater agmatine. Such high levels in the stomach may be a result of the high content of agmatine found naturally in plant and food sources (Molderings and Haenisch, 2012). The brain concentration was relative low compared to all other tissues but 5-fold greater than that detected in plasma. It was notable that a potential strain difference was observed between Long-Evans and Sprague Dawley rats with elevated concentrations of agmatine in Long Evan's rat brain at specific time points (Table 3.1) (Raasch et al., 1995). It is worth noting that we (Speltz-Paiz and Fairbanks, unpublished observations) and others (Yoon et al., 1999) have observed a significant reduction in the susceptibility of Long-Evans rats to establish nerve injury-induced hyperalgesia, which is also reduced by exogenous agmatine.

A different study quantified agmatine levels in cultured astrocytes of day old Sprague Dawley rats (Regunathan et al., 1995). The levels detected in isolated astrocytes

were greater than that observed in most rat brain samples (Table 3.1). Such an elevation could suggest that astrocytes are an important site of synthesis and/or storage of agmatine; alternatively, cultured astrocytes may not sufficiently reflect the *in vivo* condition and culture conditions may result in an elevated concentration of agmatine in the *in vitro* versus *in vivo* state. From our initial HPLC studies we reported detection of agmatine in mouse spinal cord at a level of 0.96 μg/g, substantively higher than in the rat (Fairbanks et al., 2000), which may reflect a species difference. It should be noted that the method used by Fairbanks et al. (2000) used naphthalene dicarboxaldehyde (NDA); a fluorescent compound that is more stable than *ortho*-phthalaldehyde (OPA) used in other methods and may have contributed to higher detectable levels of agmatine. However, our observations since the time of that publication suggest that putrescine may have also contributed to that result, a confound that will be discussed later.

Between 2002 and 2005 our research group conducted a study of spinally delivered agmatine to determine the spinal pharmacokinetic profile of exogenously delivered agmatine (Roberts et al., 2005). It was determined that the residence time of agmatine in spinal cord tissue significantly exceeds that of plasma. The half-life of agmatine in serum or plasma following systemic injection is typically on the order of minutes (Raasch et al., 2002; Nguyen et al., 2003; Piletz et al., 2003). However, the half-life of intrathecally injected agmatine in spinal cord was calculated to be approximately 12 hrs. This finding supports the concept that agmatine may be taken up into a cellular compartment (i.e. neurons or astrocytes) rather than effluxed to the systemic circulation. This posited pathway is supported by the work of Goracke-Postle and colleagues (2007b)

who have demonstrated transporter-dependent uptake of radiolabeled agmatine in both neurons and glia (Goracke-Postle, 2007a).

During the time of the agmatine spinal pharmacokinetic study (Roberts et al., 2005; Roberts, 2007), concern was raised in regard to the proximity of the eluted agmatine from putrescine peaks by the HPLC. It was suggested that many of the previously reported values in literature might have been attributable to putrescine rather than agmatine. Our bioanalytical studies have since been able to distinguish between agmatine and putrescine peaks in chromatographs by modifying the method from Roberts (2007) as described below.

Challenges in Limit of Detection

In order to determine the impact of overexpressed human ADC in peripheral (dorsal root ganglion) and central (spinal cord, choroid plexus) nervous system tissues where we have confirmed mRNA expression, we previously attempted to analyze extracted tissues for agmatine content by the following methods: HPLC (Roberts et al., 2005; Roberts, 2007) capillary electrophoresis (Song et al., 2004; Zhao et al., 2006), and liquid chromatography-tandem mass spectrometry (Song et al., 2004). All samples had been flash frozen (also called snap-frozen) by immersing the sample in liquid nitrogen and then storing in a -80°C freezer.

The previous HPLC-based attempts to quantify endogenous levels of agmatine in tissue samples that had been snap-frozen detected high quantities of putrescine (nmol/mg tissue), rather than agmatine, at the expected time peaks (Braun and Fairbanks,

unpublished observations). These observed levels of putrescine have been previously shown to interfere with the analysis (Braun and Fairbanks, unpublished observations). Therefore, our previous sample preparation and HPLC conditions were not able to reliably detect endogenous agmatine below the nanomol/mg tissue level. This matter has since been resolved by changing the mobile phase ratio from 65:35 acetonitrile (ACN): phosphate-buffered saline (PBS) to 80:20, and also by increasing the concentration of sodium cyanide (NaCN) and NDA used in the derivatization in Step 5 of the method in Figure 3.3. Agmatine and putrescine standards are now baseline separated (Figure 3.4). Slight variances in retention times (+/- 0.5 minutes) have been noted when switching to a new column however, the NDA-agmatine derivative is consistently eluted prior to NDA-putrescine (Churchill and Fairbanks, unpublished observations).

When spinal cord or dorsal root ganglion (DRG) from individual or pooled AAV5-hADC-treated animals were extracted, snap-frozen, and then subsequently probed by capillary electrophoresis (CE), agmatine was not detected. Again, the metabolite of agmatine, putrescine, was detected (LOD = 15 nM) (Braun and Fairbanks, unpublished observations). Finally, attempts to measure agmatine by liquid chromatography-tandem mass spectrometry (LC-MSMS) (Song et al., 2004) resulted in two different confounds. The first was that of matrix-mediated signal suppression (Braun and Fairbanks, unpublished observations). A second confound was noted, that derivatized arginine spontaneously decarboxylated at the electrospray source and, therefore, was detected as agmatine by the multiple reaction monitoring (MRM) transitions (Braun et al., unpublished observations). Therefore, these data indicate that future analyses by LC-

MSMS should include diligent method development for separation of arginine and agmatine.

Taken together, these data suggested to us that the expected elevation in agmatine concentrations following treatment with hADC could have been ultra-localized and limited to the CNS tissue populations transduced (e.g., synaptic densities or extrasynaptic astrocytic structures). Thus, any elevation of endogenous agmatine arising from the AAV5-hADC treatment may have been degraded to concentrations below our limit of detection in the samples collected prior to the discussed modified method of Roberts (2007).

Heat Stabilization

It is increasingly recognized that following conventional tissue extraction approaches (snap-freezing in liquid nitrogen, followed by -80°C storage), the extracted tissues are subject to rapid metabolism when thawing to room temperature, due to exposure to proteases and other protein metabolic enzymes (Goodwin et al., 2011). The measurement of analytes in conventionally collected tissue represent a mix of *in vivo* and *ex vivo* degradation products. Therefore, the bioanalytical signature collected post-mortem may not reflect the *in vivo* state and may result in significant sample variation or incorrect analyses (Svensson et al., 2009).

To address this confound, a technology was introduced to apply heat stabilization to freshly extracted tissues instead of snap freezing. The method of heat stabilization was introduced in 2009 (Svensson et al.) by a proteomics research group at Uppsala University. This group developed an instrument called the Stabilizor™ T1 (Denator AB,

Uppsala Sweden). The concept of the technology is that by rapidly and uniformly heating the freshly extracted tissue, degradation or metabolism is permanently and immediately stopped (Denator AB, 2014). Other researchers using this technology have found that treatment by heat stabilization with the Stabilizor™ T1 has prevented degradation of peptides in CNS tissue allowing for higher identification rates of murine brain neuropeptides such as met- and leu-enkephalin and dynorphin A and B in rat dorsal striatum (Petruzziello et al., 2013). Karlsson et al. (2013) detected 103 peptides, which included substance P, neurokinin and VGF in neonatal rat striatum that were not formerly detectable with snap-frozen tissues. Zhang et al. identified 206 peptides in a single mouse brain (2012). These peptides included neurotensin, neurokinins, enkephalins, and neuropeptide Y, among others (Zhang et al., 2012). In work by Colgrave and associates, the profiling of neuropeptides in bovine hypothalamus determined that heat stabilization with the Stabilizor™ T1 reduced interference of endogenous peptide determination because it prevented degradation of peptides, thereby reducing the amount of peptide fractions in the sample matrix (2011). Spinal cord analysis by Su et al. (2014) discovered 36 full-length, well-characterized neuropeptides such as enkephalins, nociceptin and neoendomorphins. Additionally, 118 novel spinal neuropeptides were identified as a result of implementing this new technology into their sample preparation method (Su et al., 2014).

This technique has also been used for the detection of small molecules in mouse brain such as: raclopride (347.2 g/mol) (Goodwin et al., 2011; Goodwin et al., 2012), adenosine triphosphate (MW = 507.18 g/mol), adenosine diphosphate (MW = 427.2

g/mol) and adenosine monophosphate (MW = 347.2 g/mol) (Blatherwick et al., 2013). Therefore, it was posited that this instrument could be used on our extracted mouse spinal cord to prevent the decomposition of agmatine into putrescine.

MATERIALS AND METHODS

Animals

All of the 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). Subjects were housed in groups of 4 mice 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.

Intrathecal injections

Agmatine and putrescine were purchased from Sigma Chemical (St. Louis, MO) and diluted in 0.9% NaCl. AAV5-hADC, agmatine, putrescine, or saline were administered intrathecally (i.t.) by direct lumbar puncture in conscious mice as described for mice by Hylden and Wilcox (1980). Briefly, the subjects were gently gripped by the iliac crest and a 30-gauge, 0.5 inch needle (mice) connected to a 50-µL Luer-hub Hamilton syringe was used to deliver injectate into the intrathecal space. In the case of viral vector injection, a modified needle, catheter, syringe apparatus was used to inject 10 microliters of injectate as described in Chapter 2 in order to conserve product.

Heat Stabilization vs. Snap-Freezing Comparison Study

To determine the effects of heat stabilization versus that of snap-freezing, a comparison study was performed (Figure 3.5). A total of 26 mice (ICR, Harlan, WI) of the same age and weight (21- 24 grams) were used. Ten animals were given a 5μl intrathecal (i.t.) injection of either sterile agmatine (120 nmol/5μl) or putrescine (120 nmol/5μl). Additionally, six animals were given injections of sterile saline i.t. as controls. Each animal was then sacrificed 1 minute post-i.t. injection and spinal cord harvested via hydraulic extrusion using a blunt-edged needle and syringe with cold 0.9% saline (Meikle and Martin, 1981). Immediately post-extraction, half of the samples were snap-frozen and the remaining half was heat-stabilized using the Denator Heat Stabilizer™ T1 (Uppsala, Sweden). For clarification, snap-frozen samples were processed by immediately placing the extracted spinal cord in a 1.5 ml sample vial plastic and submerging it in liquid nitrogen. The sample in the vial was placed in the -80 °C freezer until day of HPLC analysis. Heat-treated samples were placed onto specially designed cartridges (Maintainor® Tissue, Denator AB) and processed using the default "Quick Fresh Compress" method on the Stabilizor™ T1 instrument. This method uses vacuum to evacuate air from the sample area (5-10 mbar) and compresses the sample between two heating plates, and rapidly heats the sample to 90°C (does not exceed 95°C). This process takes less than 30 seconds to complete. The heat-treated samples remain in the cartridges and are placed in the -80°C until the day of HPLC analysis.

Tissue Preparation for the Comparison Study

The day of HPLC analysis, the samples were removed from -80°C storage and 10-25 mg of spinal cord was weighed in a new 1.5 ml sample vial and prepared for derivatization and injection onto the HPLC according to the method outlined in Figure 3.3. Briefly, the samples were acid-precipitated with perchloric acid (150 µL, 0.6 N) and hydrochloric acid (65µL, 0.1 M in methanol) and allowed to sit on ice for 30 minutes. After 30 minutes, the solution was neutralized with KOH (4.2M) to a pH of approximately 7.0-7.5 by dotting on pH paper. Once neutralized, the samples were centrifuged (Eppendorf, Model: 5415 D) at 10,000 rpm (9280 x g) for 15 minutes. Approximately 280 µL of the aqueous phase was transferred to a second set of 1.5 ml vials and allowed to sit over night in the -20°C freezer. The next day, the samples were centrifuged at 10,000 rpm (9280 x g) for 15 minutes. A salt pellet from the neutralization formed in the bottom and 250 µL of the aqueous phase was transferred to a third set of vials. This final supernatant was finally concentrated to dryness by vacuum centrifugation (Savant, Speed Vac® Model: SC100).

HPLC Analysis for the Comparison Study

The dried and concentrated samples from the aforementioned procedure were re-suspended in 100 µL borate buffer (pH 9.4) and derivatized with sodium cyanide (100 µL, 0.025 M) and naphthalene dicarboxaldehyde (NDA, 200 µL, 0.01 M in methanol). This solution was allowed to react at room temperature for approximately 30 minutes,

producing an agmatine-NDA derivative of high fluorescence and stability for HPLC analysis (Hewlett-Packard, 1100 Series).

An injection volume of 20 μL was used for all samples and standards at 25ºC (Hewlett-Packard Column Bank Model: G1316A and Autosampler Model: G13129A) and injected onto a 250 x 4.6 mm, Alltima C8, 10 micron column (Grace Davison Discovery Science, Deerfield, IL) at a flow rate of 1.5 mL/min (Hewlett Packard, Quaternary Pump Model: G1311A). The mobile phase consisted of 80% acetonitrile (HPLC Grade): 20% phosphate-buffered saline (3.42 g monopotassium phosphate and 4.32 g dipotassium phosphate in 1 L H_2O) (pH: 6.81) and was filtered prior to every run or sequence through a 0.44 μm nylon filter. A degasser unit (Hewlett-Packard Model: G1322A) was also employed.

The fluorescence intensity of NDA-agmatine and NDA-putrescine derivatives were recorded over time using an excitation wavelength of 249 nm and an emission wavelength of 466 nm (Hewlett-Packard Model: 1046A). Agmatine and putrescine standards were run concurrently in order to obtain the most accurate estimates of tissue and serum agmatine concentrations. Evaluation of peak retention time and peak area was conducted with the HP Chemstation software (Ver. 10.02). During method refinement, external spikes of agmatine standards were employed to verify the NDA-agmatine peak in the chromatograph (Figure 3.6).

Modified HPLC Analysis of Agmatine in Mice Receiving AAV5-hADC

Early HPLC method development evaluated different ratios of mobile phase (acetonitrile (ACN) and phosphate-buffered saline (PBS)) and their effect on agmatine and putrescine standard retention time and peak area. It was determined that a ratio of 80:20 ACN:PBS achieved consistent baseline separated peaks for agmatine and putrescine (Figure 3.4) (Churchill and Fairbanks, unpublished observations). Thus, all work described here uses the 80:20 ratio for the mobile phase, not the 65:35 as described by Roberts et al. (2005), regardless of tissue preparation technique.

Only in the studies quantifying agmatine levels in AAV5-hADC-treated tissue, was the Stabilizor™ T1 used to prepare extracted spinal cords, instead of snap-freezing, in order to inactivate any metabolic activity that could potentially degrade agmatine to an undetectable level. In addition to using heat stabilization, the aforementioned HPLC method was also modified when analyzing the tissue from the AAV5-hADC study (Chapter 2, Figure 2.2) by excluding methanol from the reagents and solvents used in the tissue preparation. Specifically the 0.1M HCl solution in methanol was omitted and the NDA was dissolved in acetonitrile to prevent methanolysis of our primary analyte, agmatine. Therefore it was determined collectively that the heat stabilization of the tissue, coupled with the combination of the modified mobile phase, and removal of methanol from the derivatization step, would prevent degradation of agmatine, our compound of interest, thereby enabling the detectability of agmatine in mice spinal cord tissue.

RESULTS

Heat Stabilization vs. Snap-Freezing Comparison Study

A primary goal of the study was to determine whether heat stabilization improved the limit of detection for agmatine in our HPLC-based bioanalytical assay. To address this question we conducted a study to compare mouse spinal cord levels of agmatine and putrescine where the extracted samples were either snap-frozen (liquid nitrogen) or heat-stabilized (Stabilizor™ T1) (Figure 3.5). Because the previous HPLC method had been shown to detect levels of injected agmatine i.t. (Roberts et al., 2005), it was decided for this study to inject agmatine (120 nmol), putrescine (120 nmol) or saline by direct lumbar puncture. Spinal cord tissue was extracted and collected 1 minute post-injection.

Figure 3.7A shows agmatine levels in spinal cords treated with either of the aforementioned treatments. The first two bars illustrate that agmatine detection was approximately the same in snap-frozen or heat-treated samples treated with exogenous agmatine. Average values for bars 1 and 2 ("i.t. Ag-Snap" and "i.t. Ag-Heat") were 0.036 nmols/mg tissue and 0.036 nmol/mg tissue, respectively. Bars 3 and 4 ("i.t. Put-Snap" and "i.t. Put-Heat") show that apparent reduction of agmatine was not statistically significant in heat-treated subjects as compared to those samples that were snap-frozen. The average agmatine value for snap-frozen (putrescine i.t.) was 0.0045 nmol/mg tissue and 0.0010 nmol/mg tissue in heat-treated (putrescine i.t.). Finally, bars 5 and 6 ("i.t. Saline-Snap" and "i.t. Saline-Heat") illustrate a limited detection of endogenous agmatine in spinal cords from mice intrathecally injected with saline regardless of snap-freezing or heat-treatment. The average agmatine in snap-frozen (saline i.t.) was 0.00065 nmol/mg

tissue. The average agmatine value in heat-treated, (saline i.t.) was 0.00098 nmol/mg tissue.

Figure 3.7B depicts spinal putrescine levels in spinal cords treated with one of the three aforementioned treatments. The first two bars ("i.t. Ag-Snap" and "i.t. Ag-Heat") illustrate that putrescine detection was significantly reduced (Student's t-test, $p<0.005$) in heat-treated samples compared to snap-frozen samples that were treated with i.t. agmatine. The average putrescine value for snap-frozen (agmatine i.t.) was 0.11 nmol/mg tissue and 0.0.039 nmol/mg tissue in heat-treated (agmatine i.t.). Bars 3 and 4 ("i.t. Put-Snap" and "i.t. Put-Heat") show that the apparent reduction of putrescine was not statistically significant in heat-treated samples as when compared to those that were snap-frozen that were treated with exogenous putrescine. The average putrescine values for snap-frozen and heat-treated (putrescine i.t.) were 0.27 and 0.14 nmol/mg tissue, respectively. Finally, bars 5 and 6 ("i.t. Saline-Snap" and "i.t. Saline-Heat") illustrate a limited detection of endogenous putrescine in spinal cords from mice intrathecally injected with saline regardless of snap-freezing or heat-treatment. The reduction of putrescine in heat-treated (saline i.t.) was statistically significant (Student's t-test, $p<0.05$) when compared to snap-frozen (saline i.t.). The average putrescine values for snap-frozen and heat-treated (saline i.t.) were 0.468 and 0.061 nmol/mg tissue, respectively. The levels of exogenously delivered agmatine in spinal cord tissue seemed unaffected by heat stabilization; however, heat-treatment reduced putrescine in the sample matrix, whether derived from the conversion of agmatine by agmatinase, or by other degradative processes. This was interpreted as an improvement for our method development.

Therefore, this instrument was used to preserve all subsequent tissue extractions, including those from the AAV5-hADC studies. It was expected that with less putrescine present in the tissue sample, the derivatization reaction in Figure 3.2B would enable all agmatine to be labeled with NDA. Any agmatine present would not have to compete with high levels of putrescine to be reacted with NaCN and labeled with NDA.

Acetonitrile vs. Methanol: Influences on Agmatine Detection

Prior to the presentation of the bioanalytical results from the AAV5-hADC study, it is valuable to present an additional refinement that has been made to the HPLC method. After extensive literature review on previous agmatine detection via HPLC-fluorescence, it was discovered that the labile nature of agmatine might be due in part to decomposition by methanolysis (Wehr, 1995). That is, when agmatine is in the presence of methanol, agmatine can readily break into fractions (Wehr, 1995).

Previous to this finding, all tissue samples had been processed with methanol in two different steps of the method described in Figure 3.3. First in step 2, the acid precipitation step, a solution of perchloric and hydrochloric acid in methanol is used. Second, methanol is used in the fifth step (Figure 3.3.) to dissolve the fluorescent label for primary amines, napthalene dicarboxyaldehyde (NDA). It was a concern that perhaps previous attempts to detect agmatine in samples had been subject to methanolysis as described by Wehr (1995). To quickly compare the influence of methanol on the detection of agmatine, an experiment was conducted using agmatine and putrescine standards in water. One set of standards was made for each compound and divided in

half. One half to be prepared using methanol and the other half to be prepared with acetonitrile (HPLC grade). Acetonitrile was chosen due to similar polarity as methanol.

The peak area and concentration were plotted and the R-squared values and peak area were compared (Figure 3.8). The standards that used methanol in their preparation had the following R^2 values: 0.848 for agmatine and 0.994 for putrescine. The standards that used acetonitrile instead of methanol had the following R^2 values: 0.995 for agmatine and 0.975 for putrescine. It was also observed that the agmatine standards that used acetonitrile had slightly less peak area compared to the same standards that used methanol (Figure 3.8). Peak areas in the putrescine standards were also lower in the acetonitrile group as compared to the those derivatized with methanol. These results suggest that with the current column and modified HPLC method, a methanol-free sample preparation would yield more accurate detection of agmatine.

Heat-Stabilized Assessment of Agmatine in hADC-Treated Mice

Based on the previous results, it was decided that the HPLC method would be modified when analyzing the tissues collected from animals in the AAV5-hADC studies. For all studies of tissue extracted from AAV5-hADC treated subjects, the method would be refined to use the Stabilizor™ T1 at the time of tissue collection and the subsequent sample preparation and derivatization of the tissue would be conducted in a methanol free manner. The combination of using the Stabilizor™ T1 and acetonitrile (instead of methanol) would reduce levels of putrescine from the sample matrix and eliminate any possibility of methanolysis of agmatine. These two changes assisted in enabling lower levels of agmatine to be more detectable.

To determine whether hADC expression resulted in increased levels of agmatine in tissue, we hydraulically extruded and heat-treated the spinal cords from mice treated with AAV5-hADC (n=14) and saline-treated controls (n=6). Only the cervical portion of the spinal cord was analyzed by HPLC because in several cohorts, more mice appeared to have hADC expression in the cervical spinal cord region. These mice were among those used for the studies conducted in Chapter 2. Consistent with the hypothesis, the cervical spinal cords of mice that had received AAV5-hADC pre-treatment contained significantly higher levels of agmatine than their saline counterparts (Student's t-test $p<0.05$) (Figures 3.9 and 3.10).

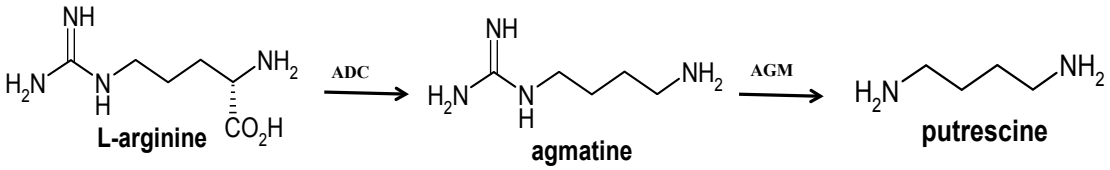
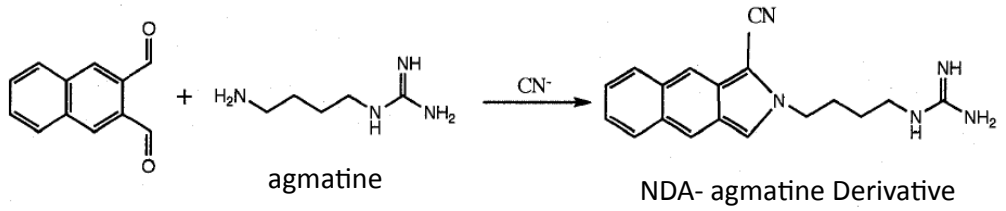


Figure 3.1: Agmatine pathway: Agmatine is synthesized by converting L-arginine via arginine decarboxylase (ADC) into agmatine and agmatine is metabolized by agmatinase (AGM) into putrescine.

A



B

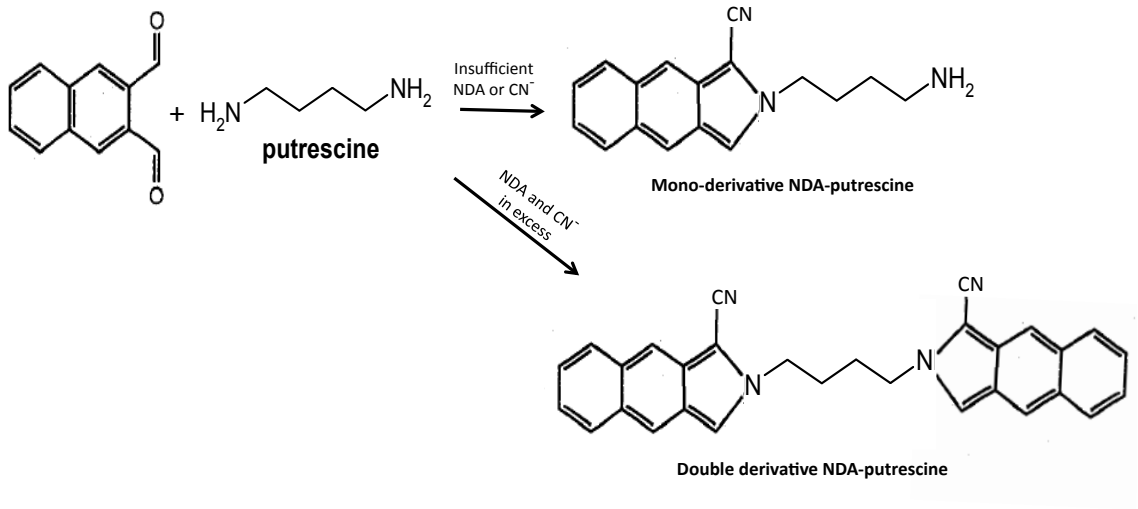


Figure 3.2: (A) Derivatization reaction of napthalene dicarboxaldehyde (NDA) to the primary amine group of agmatine in the presence of cyanide. **(B)** Insufficient concentration of NDA and cyanide could cause a confound with the mono-derivative of NDA-putrescine since it will have a similar retention time as the NDA-agmatine. This method provides excess NDA and cyanide to prevent this from happening.

Tissue	Species (n)	Reported Concentration (ng/g ww)	Conversion to pmol/g ww (Based on mw of 130.192)	Detection Method	Reference
Rat Brain (Region not noted)	Sprague Dawley, 5 months	2.40 ± 0.60	18	HPLC, OPA Derivatization	Raasch et al., 1995
Rat Brain Cortex	Long Evans 3 months	1.25	9.6	HPLC, OPA Derivatization	Raasch et al., 1995
	Long Evans 14 months	1.55	11.9	HPLC, OPA Derivatization	Raasch et al., 1995
	Long Evans 24 months	8.3	63.7	HPLC, OPA Derivatization	Raasch et al., 1995
Rat Brain Cerebellum	Long Evans 3 months	1.06	8.14	HPLC, OPA Derivatization	Raasch et al., 1995
	Long Evans 14 months	1.45	11	HPLC, OPA Derivatization	Raasch et al., 1995
	Long Evans 24 months	9.4	72.2	HPLC, OPA Derivatization	Raasch et al., 1995
Cultured Primary Astrocytes	Sprague Dawley Postnatal Day 1	8.5	65	HPLC, OPA Derivatization	Regunathan et al., 1995
Mouse Spinal Cord	Mouse ICR-CD1	0.96 ± 0.14 (*micrograms/ gram)*	7300	HPLC, NDA Derivatization	Fairbanks et al., 2000

Table 3.1: Levels of endogenous agmatine in murine tissues of the central nervous system. (Abbreviations: OPA, *ortho*-Phthaladehyde; NDA, naphthalene dicarboxyaldehyde; ww; wet weight)

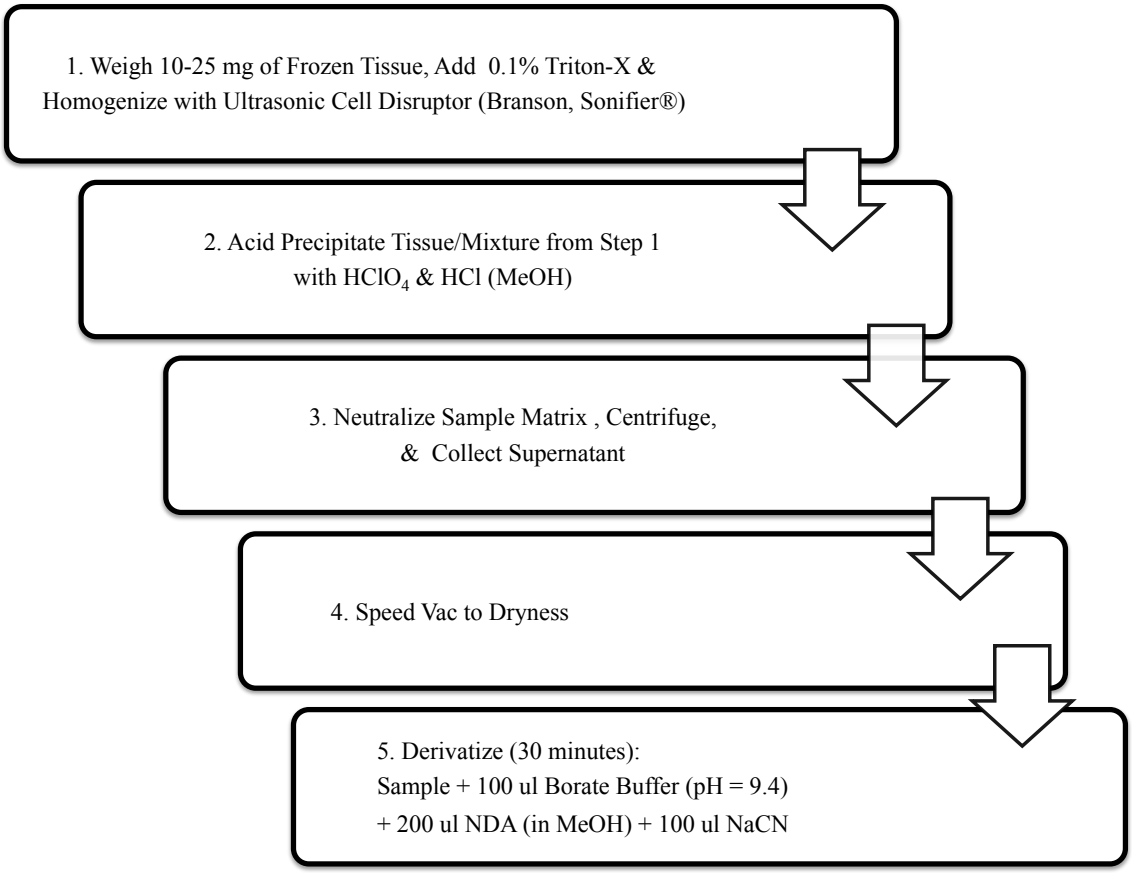


Figure 3.3: Overview of tissue preparation and derivatization of sample for HPLC analysis. (Abbreviations: MeOH, methanol; NDA, napthalene dicarboxaldehyde)

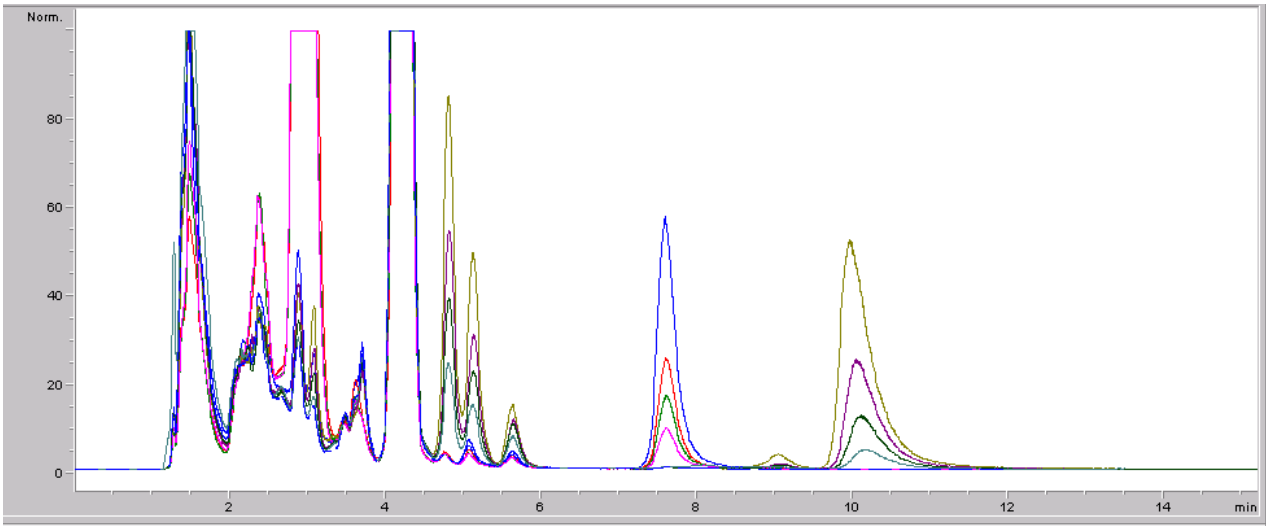


Figure 3.4: Chromatographs of agmatine and putrescine standards in water. Standards were derivatized with excess sodium cyanide (NaCN) and NDA and run with a mobile phase of 80:20 acetonitrile (ACN): phosphate-buffered saline (PBS). For this particular sequence, on this particular column, the average retention times for the NDA-agmatine derivative was 7.6 minutes and 10.1 minutes for the NDA-putrescine derivative. Agmatine standards in the chromatograph are in order of decreasing concentration: 12.5, 6.25, 3.125, 1.5625 and 0 picomols/injection. Putrescine standards in the chromatograph are also in order of decreasing concentration: 200, 100, 62.5, 25, and 0 picomols/injection.

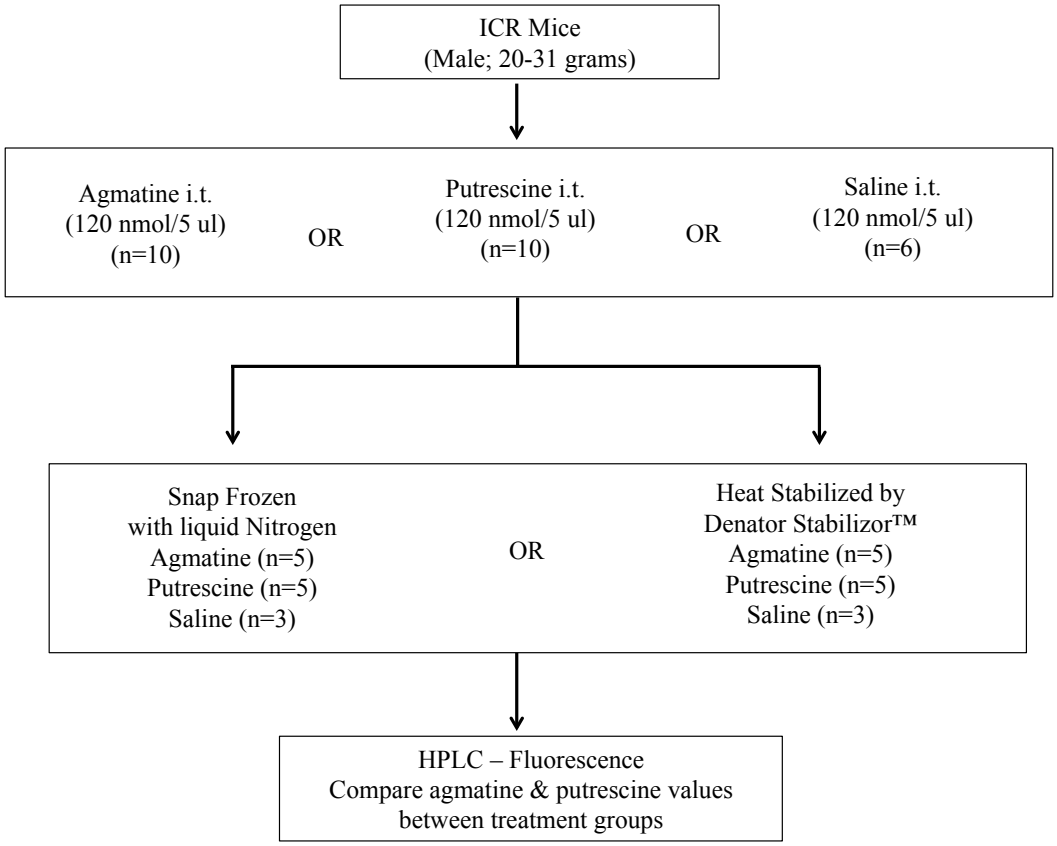


Figure 3.5: Comparison Study Overview. Mice were given an inrathecal (i.t.) injection of (sterile) agmatine, putrescine or saline. Half of the samples were snap-frozen by submersion in liquid nitrogen whereas the other half was heat-stabilized with the Stabilizor™ T1 Instrument.

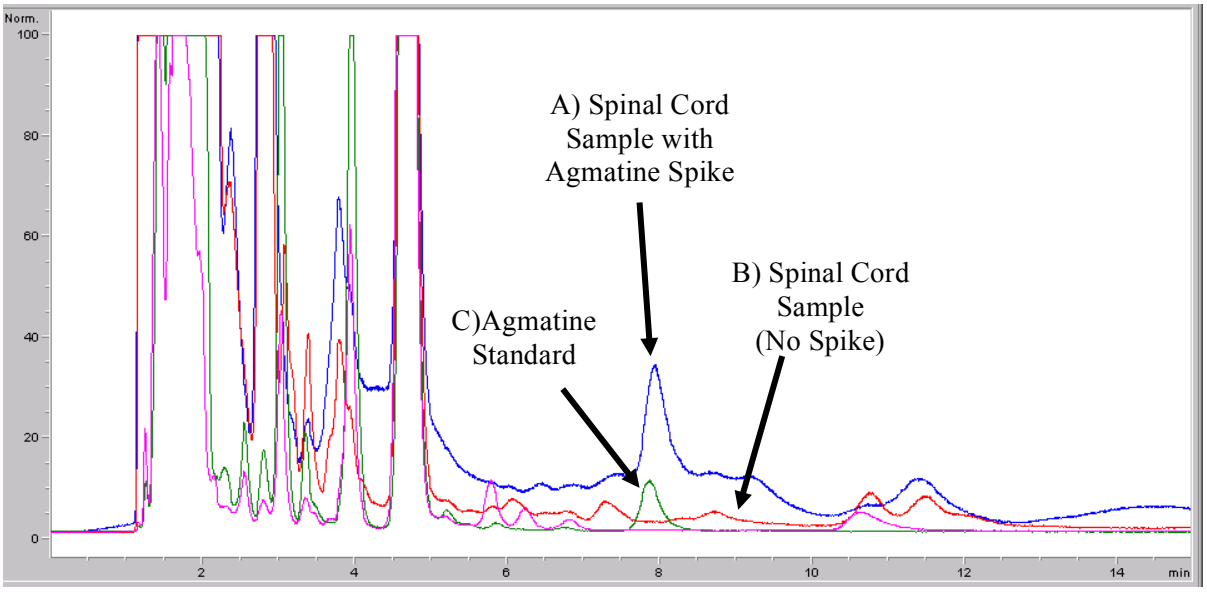


Figure 3.6: Representative chromatographs of A) spinal cord tissue spiked with agmatine B) spinal cord tissue with no agmatine spike and C) agmatine standard in water (12.5 picomols/injection). The sample of spinal cord that was spiked with agmatine (A) has an approximate peak retention time of 7.8 minutes. This is comparable to the retention time of the peak of agmatine standard in water (C) that was also approximately 7.8 minutes. The spinal cord that received no agmatine spike (B) showed no detectable amounts of agmatine.

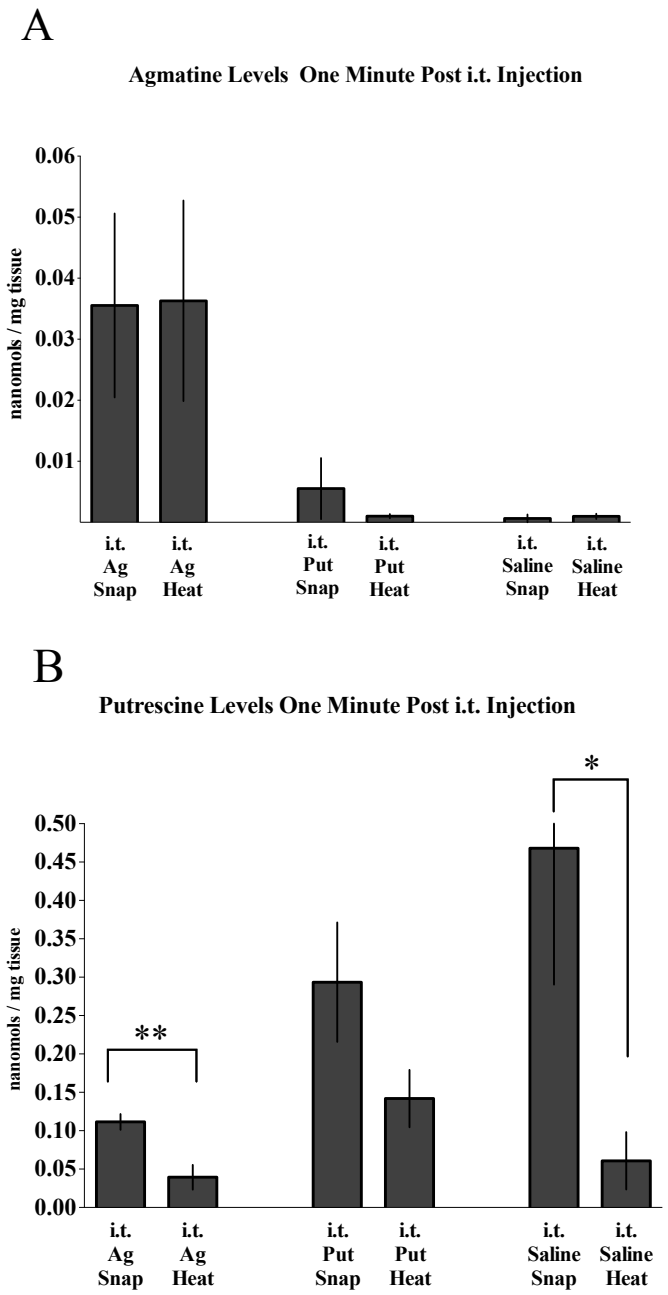
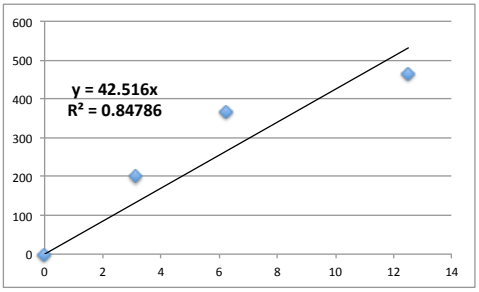


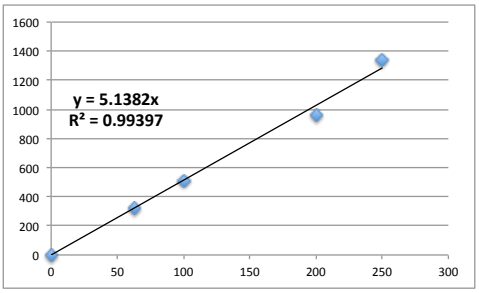
Figure 3.7: Comparison Study: Snap-Frozen Spinal Cord vs. Heat-Stabilized Spinal Cord. **(A)** Levels of agmatine were not significantly different across groups and tissue preparation technique. **(B)** Levels of putrescine were decreased using the heat treatment technique in animals receiving i.t. agmatine and i.t. saline, but not i.t. putrescine. (significant difference $^{**}p<0.005$ and $^{*}p<0.05$, Student's t-test) Methanol used in preparation and derivatization.

Methanol Standards

Agmatine	
pmol/20 ul	Area
12.5	464.8
6.25	365.4
3.125	200.1
0	0

Putrescine	
pmol/20 ul	Area
250	1337
200	964.8
100	507.5
62.5	322.6
0	0

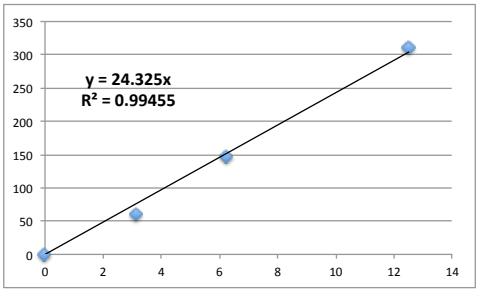




Acetonitrile Standards

Agmatine	
pmol/20 ul	Area
12.5	310.3
6.25	147.2
3.125	60.7
0	0

Putrescine	
pmol/20 ul	Area
250	186.8
200	157
100	60.2
62.5	26.28
0	0



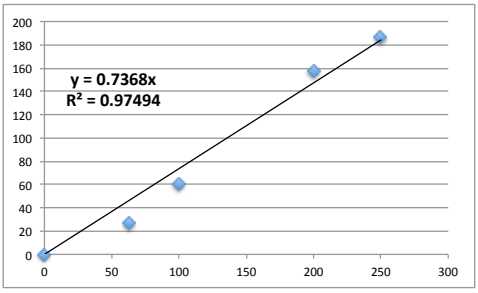


Figure 3.8: Agmatine and putrescine standards derivatized with NDA in methanol (top) or acetonitrile (bottom) for 30 minutes then injected onto HPLC.

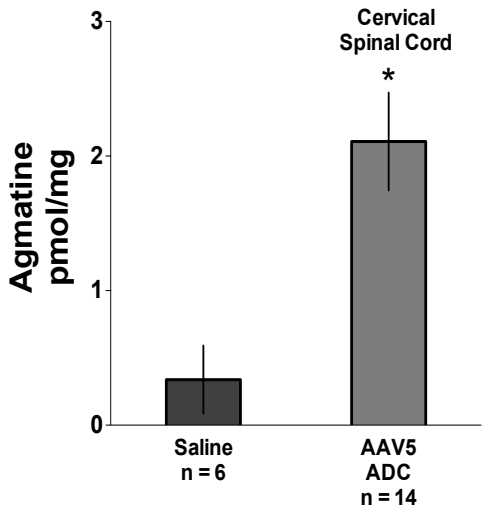


Figure 3.9: Agmatine levels in heat-protected cervical spinal cord were elevated in AAV5-hADC treated mice relative to saline-treated controls. Sample preparation and derivatization was methanol free. (*significant difference; $p<0.05$, Student's t test)

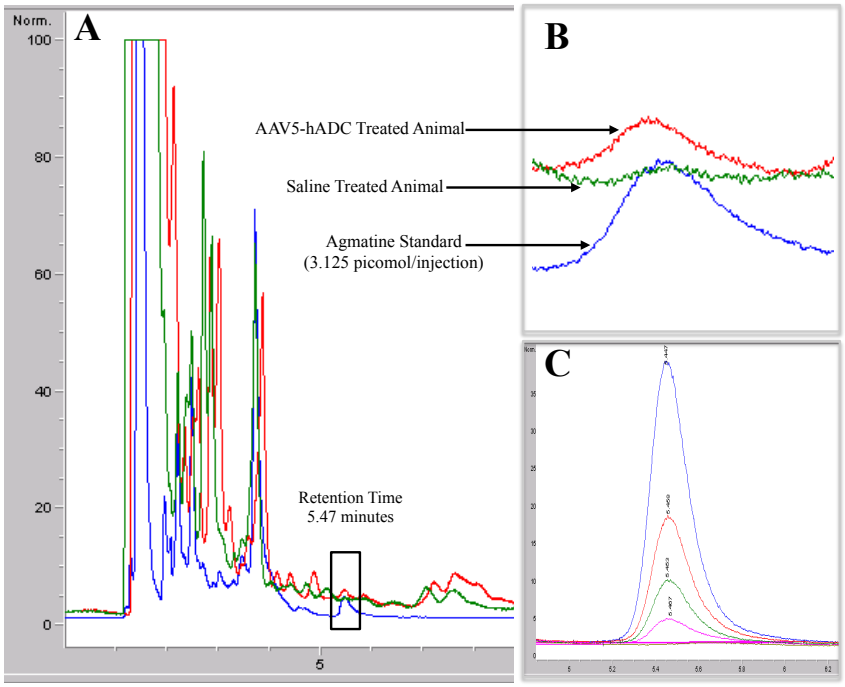


Figure 3.10: Chromatographs from AAV5-hADC Study: **A)** Overlay of spinal cord tissue; animal treated with AAV5-hADC i.t. (red), animal treated with saline i.t. (green), and agmatine standard (3.125 picomols/injection) (blue). Square represents region where agmatine standards elute (retention time of 5.5 minutes). **B)** Enlargement of enclosed area at 5.5 minutes from panel A: spinal cord from an animal treated with AAV5-hADC i.t. (red), spinal cord from an animal treated with saline i.t. (green) and the 3.125 picomol/injection standard (blue) **C)** Overlay of agmatine standards in order of decreasing concentration: 25, 12.5, 6.25, 3.125 and 0 picomols/injection.

DISCUSSION

Heat-Stabilizing vs. Snap-Freezing

Typical tissue harvesting involves "snap-freezing" of extracted tissue. This is frequently performed by submerging samples in liquid nitrogen or dry ice to stop any metabolic processes taking place in the extracted tissue. The goal is to preserve the extracted tissue in a state that is as close as possible to the *in vivo* environment. An alternative to snap- freezing samples is to denature proteins and enzymes by heating, such as with microwave (O'Callaghan and Sriram, 2004). Laboratory microwaves and their animal retainers are specifically designed for rapid preservation of the intact murine brain, and would not accommodate spinal cord preservation. Currently, there is no such device for microwaving spinal cord *in vivo* (personal correspondence with manufacturer, Ugo Basile).

Prior to 2009, instrumentation for the preservation of murine spinal cord tissue or other tissue and organs (up to 7 mm in thickness) was not available. Denator AB, has filled this gap in technology with their Heat Stabilizer™ T1 instrument. For this research, the mouse spinal cord was hydraulically extruded (Meikle and Martin, 1981) and immediately placed onto specially designed cartridges (Maintainor® Tissue). This cartridge is placed in a holder on the Stabilizor™ T1 instrument. Once the start button has been pressed, air is evacuated from sample area and the instrument proceeds to rapidly heat denature the sample. A Mass Spectrometry Imaging study has shown a significant difference in (AMP) and (ATP) levels in brain preserved via snap-freezing versus heat-stabilized (Goodwin et al., 2011; Goodwin et al., 2012). This difference is

believed to be due to the metabolic enzymes and proteins that resume their degradation activity when the sample is removed from -80°C, thawed, and the temperature increases. Consequently, ATP levels are elevated in heat-stabilized relative to snap-frozen and, as expected, AMP levels are reduced in heat-stabilized compared to snap-frozen. Rapid degradation has been reported to take place in snap-frozen samples in intervals as short as 5 minutes at room temperature (Goodwin et al., 2011).

For a molecule such as agmatine, the concern with snap-freezing and thawing of tissue samples, is that agmatine could readily be metabolized into putrescine via agmatinase. Therefore, in hopes of optimizing the HPLC limit of detection of agmatine in mouse spinal cord, a comparison study between snap-frozen (liquid nitrogen) and heat-stabilized (Stabilizor™, Denator AB, Uppsala, Sweden) samples was conducted (Figure 3.5). This study was conducted by exogenously delivering agmatine (120 nmol/5 μL) or putrescine (120 nmol/5 μL) or saline by direct lumbar puncture. These values were chosen based on previous data showing reliable HPLC detection of exogenously delivered agmatine (Roberts, 2005; Roberts, 2007). It must be noted, that this comparison study used the tissue preparation and sample derivatization that included the use methanol, therefore the levels of agmatine reported in the heat-treated vs. snap-frozen study (Figure 3.7) may be lower than actual values due to methanolysis as described below in the next section. This may explain why there were no statistically significant differences in agmatine concentration following exogenous delivery of i.t. agmatine, putrescine or saline, between heat-stabilized and snap-frozen treatment. It could also be that the high concentration of i.t. agmatine resulted in higher, overall concentrations of

detectable agmatine, potentially obscuring any loss due to metabolism. Another consideration is that the degradation of endogenous L-arginine into agmatine was contributing to overall agmatine levels in the snap-frozen samples. In future studies, the use of i.t. injected L-arginine would be of interest to confirm the endogenous capability of overexpressed arginine decarboxylase to generate agmatine.

Although there were no discernable differences in the agmatine levels between heat-treated or snap-frozen, this study did reveal that two groups of snap-frozen tissues had statistically significant more putrescine, the metabolite of agmatine, than the heat-stabilized samples (Figure 3.7B). This was observed in the i.t. agmatine and i.t. saline groups. It was somewhat surprising that the levels of putrescine in snap-frozen, i.t. saline animals were greater than that of both snap-frozen and heat-treated i.t. putrescine. However, this may be attributable to the lower group size for the i.t. saline animals (n=2 for snap-frozen; n=3 for heat-treated) than in the i.t. agmatine and i.t. putrescine animals (n=5 for all groups). Levels of putrescine, overall, were higher in snap-frozen samples. Since putrescine is a natural compound that occurs in necrotic tissue, it could be that other decompositional pathways are contributing to the increased levels of putrescine in the all snap-frozen tissue.

The key finding of this study was that heat stabilization significantly reduced concentrations of spinal putrescine following exogenous delivery of agmatine, presumably derived from the metabolism of exogenously delivered agmatine. One might have expected that concentrations of spinal agmatine following exogenous delivery of agmatine may have been elevated in the heat-treated versus snap-frozen samples in

Figure 3.7A, but it may be that the excessive concentrations of agmatine delivered spinally obscured a detectable difference. From that point on, the Denator Stabilizor ™ T1 would be employed to treat freshly extracted tissues. It was posited that if the metabolism or degradation of agmatine could be stopped, the levels of agmatine might become detectable with the HPLC method. At the very least it would reduce the amount of putrescine that would compete for derivatization in step five of the HPLC method. This leads to discussion of the second modification made to the HPLC method.

Methanolysis Prevention

When searching the literature regarding quantification and derivatization of agmatine, it has been described as labile (Satriano, 2004) and even "irreproducible" (Wehr, 1995). Precipitation of proteins in the biological sample with perchloric acid (PCA) in step one (Figure 3.3) is a common technique employed with biological tissue samples (Hanai, 1991). Not only does the PCA technique remove proteins, it also creates a stable environment for small molecules (Hanai, 1991). Other researchers quantifying agmatine in biological tissues have also used this technique routinely (Li et al., 1994; Regunathan et al., 1995; Feng et al., 1997; Rawls and McGinty, 1997; Hockl, 2000).

Research on the quantification of agmatine is abundant when analyzing this molecule in bacteria or plants. The most well known technique for agmatine detection in plants is by Flores and Galston (1982). Even in the plant system, issues of interfering amines and resolution of agmatine existed (Slocum et al., 1988; Slocum et al., 1989). Further investigation into the issues of agmatine quantification in plants revealed that

agmatine undergoes rapid methanolysis if the derivative is dissolved in methanol (Wehr, 1995). Methanolysis is the process of exchanging the organic group of an ester with the organic group of an alcohol. The author also notes this as the main reason why previous studies of agmatine quantification (in plants) have largely been irreproducible (Wehr, 1995).

The HPLC method published by Roberts et al. (2005 and 2007) were based on a combination of the following published methods. The isocratic mobile phase for HPLC (Feng et al., 1997), the acid precipitation of the biological tissue (Raasch et al., 1995) and finally the use of the more stable fluorescent compound naphthalene-2,3-dicarboxaldehyde (NDA) in the derivatization step (De Montigny, 1987). In the presence of cyanide, NDA will react with primary amines such as agmatine and putrescine (Figure 3.2A). It should be noted, that an excess of both the cyanide ion and NDA is required to push the derivatization process to full labeling of both primary amines of putrescine (Figure 3.2B). If insufficient amounts of NDA and cyanide are used in the derivatization, it is possible to have a monoderivative of NDA-putrescine which can be a confound with the NDA-agmatine derivative since they would have a similar retention time. Thus, excess levels of NDA (0.01 M) and NaCN (0.025 M) are recommended to label both amines sites on the putrescine molecule (Figure 3.6B) resulting in a longer retention time than the NDA-agmatine derivative Figure 3.4.

Comparing these publications to the HPLC methods developed by Roberts (2005 and 2007) the following similarities and differences were noted. For the acid-preparation step, Raasch (1995) used a combination of 0.6N Perchloric acid ($HClO_4$) and 0.1N

hydrochloric (HCl) acid *in methanol*. This is consistent with both the publication by Roberts et al, (2005) and the thesis work by Roberts (2007). Feng (1997) uses an isocratic technique for mobile phase elution. This is also consistent with both Roberts et al. (2005) and Roberts (2007). In the derivatization by De Montigney (1987), NDA was dissolved in acetonitrile. Yet, the method used by Roberts in his thesis (2007), NDA in methanol was used. Therefore, it was posited that the preparation and derivatization of tissue samples that involved methanol may have degraded agmatine in a similar manner as described by Wehr (1995) in their plant agmatine quantification.

In the study comparing a series of NDA-derivatized standards with and without methanol (figure 3.8), the R^2 value for agmatine was improved with the use of acetonitrile instead of methanol. Additionally, overall lowered putrescine peak area was observed when using acetonitrile. Agmatine, specifically in brain tissue has also been said to be subject to "overcrowding" in detection methods (Raasch et al., 1995); that is to be lost among the numerous amines present in a biological tissue sample. Since the goal of our analysis is to improve agmatine detection, using acetonitrile instead of methanol, would lower interference of putrescine and also yield more confidence in the linearity of our standards. Thus, the decision was made to use NDA dissolved in acetonitrile and remove the HCl in methanol from the acid precipitation step when determining levels of agmatine in animals treated with AAV5h-ADC.

Quantification of Agmatine with Modified Sample Preparation & Derivatization

With the new HPLC tissue preparation technique and removal of methanol from the chemical matrix, the tissues from animals treated with AAV5-hADC were processed and evaluated for agmatine levels. Agmatine levels in heat-protected cervical spinal cord were elevated in AAV5-hADC injected mice relative to saline-treated controls and statistically significant ($p<0.05$, Student's t test) (Figure 3.7). Therefore, these observations support the proposal that elevated endogenous agmatine contributes to the effect of the AAV5-ADC treatment to protect against opioid-induced analgesic tolerance featured in Chapter 2 of this thesis

SUMMARY AND CONCLUSIONS

Taken together, these results indicated that heat stabilization, applied to reduce post-mortem metabolism of extracted tissue, may enable better detection of agmatine in CNS tissue at the picomol/mg tissue level. Agmatine is known to have other therapeutic effects, thus, the heat stabilization approach may be important or valuable to other investigations of endogenous or exogenous agmatine effects in other forms of maladaptive neuroplasticity (e.g. learning and memory, stroke, spinal cord injury).

We have demonstrated that heat stabilization of extracted spinal cord reduces spinal cord putrescine concentrations following either agmatine or putrescine intrathecal injection. This procedure may be essential to prevent post-mortem metabolism of agmatine to its primary metabolite, putrescine. Our previous studies using liquid nitrogen mediated flash frozen post-mortem processing failed to sufficiently and reliable detect

and/or distinguish agmatine whether evaluated by HPLC, capillary electrophoresis, or mass spectrometry. Heat stabilization has enabled us for the first time to reliably detect agmatine in the spinal cord at the picomol/ mg tissue level via HPLC and, importantly, to demonstrate that a single intrathecal injection of AAV vector carrying the gene for arginine decarboxylase results a significant elevation agmatine concentration in extracted heat-stabilized mouse spinal cord. Heat stabilization will be used in future studies to evaluate elevation of agmatine following AAV-hADC delivery to a wide spectrum of CNS tissues (dorsal root ganglia, selected brain nuclei, choroid plexus) in mouse and in rat, and under varying conditions of neural plasticity (neuropathic pain, spinal cord injury). From these results, we believe that this approach may become broadly applied to study the agmatinergic system.

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CHAPTER 4. *Gene Transfer to Choroid Plexus Cultures*

CHAPTER SYNOPSIS

The innate ability of the choroid plexuses to produce and secrete bioactive proteins into the cerebral spinal fluid (CSF) (Gonzalez et al., 2010) makes this tissue a potential target for central nervous system gene therapy. We have previously shown that intrathecal delivery of adeno-associated viral vectors results in gene transfer to the choroid plexus (Schuster et al., 2014ab, Chapter 2 of this thesis). In order to further characterize gene transfer to the choroid plexus we have developed an *in vitro* method for evaluating gene transfer to dissociated primary choroid cells, intact choroid tissue (explants) and immortalized choroid plexus cell line, Z310 (Zheng and Zhao, 2002).

INTRODUCTION

The choroid plexus is small organ inside the ventricles of the brain. The function of this tissue is to secrete cerebrospinal fluid (CSF), which supplies the primary fluidic distribution system of the central nervous system (CNS). During the time that our research team has investigated the neuroanatomical distribution of gene expression of the adeno-associated viral (AAV) vectors we have noticed that certain AAV serotypes will transduce choroid plexus *in vivo*, while others do not. This was demonstrated immunohistochemically with visualized green fluorescent protein *in vivo* (Schuster 2014ab). In agreement with that observation, the results that I presented in Chapter 2 of this thesis show that intrathecal delivery of AAV5-hADC also results in expression of mRNA of human ADC in freshly extracted choroid tissue. Therefore, the possibility that the therapeutic effects observed from AAV5-hADC intrathecal delivery may arise from gene transfer to the choroid plexus, subsequent, agmatine production, and distribution through the CSF to target regions in the CNS. The choroid plexus may therefore present an attractive target tissue for therapeutic development as a constitutive delivery source of endogenous therapeutics. It is not yet known, but it may be, that gene transfer to the choroid plexus is sufficient (e.g. in the absence of gene transfer to neurons or glia) for therapeutic benefit. Such an approach could prove useful for a wide variety of gene therapies intended to treat a broad spectrum of CNS disorders (Johanson et al., 2005; Hester et al., 2009; Gonzalez et al., 2010; Gonzalez et al., 2011).

Although we have several reports of green fluorescent protein expression in choroid plexus cells following AAV delivery (Schuster et al., 2014a; Schuster et al.,

2014b), our understanding of the suscpetibility of choroid plexus cells to gene transfer is fairly limited. In order to assess the utility of specifically targeting the choroid plexus for gene transfer, it is important to understand its relative amenability to gene transfer particularly by the primary AAV subtypes used to target the CNS. The objective of the research featured in Chapter 4 was to quantify gene transfer to cells of the choroid plexus compared between three AAV subtypes commonly used to target the CNS: AAV5, AAV8, and AAV9 (Hester et al., 2009; Van Vliet et al., 2009; Mason et al., 2010; Dirren et al., 2014; Schuster et al., 2014a).

The Choroid Plexus

The choroid does not line the ventricle, instead it sprouts from the ventricle ependymal cell layer in the form of a cluster of epithelial cells (Johanson et al., 2005). The cluster is a composed of a single sheath or monolayer of epithelium cells with extensive foldings. Praetorius describes the morphology of this sheath as containing a "basal labyrinth" or having "basal infoldings" (2007). Chodobski concurs with this description and describes it as a "tightly packed villous folds consisting of a single layer of cuboidal epithelial cells overlying a central core of highly vascularized stroma" (Chodobski and Szmydynger-Chodobska, 2001). The layer of epithelial cells that form the choroid plexus cell layer also has tight junctions (Szmydynger-Chodobska et al., 2007; Johanson et al., 2011). The smooth, basal surface faces the microvasculature that supplies the choroid with blood. The luminar or ventricular surface facing the cerebral spinal fluid (CSF) has lush microvilli (Matsushima, 1983; Villalobos et al., 1997;

Emerich et al., 2005). The choroid and cellular attributes, including transport systems, have been compared to that of the leaky epithelia of the kidney (Emerich et al., 2005; Johanson et al., 2011; Redzic, 2011).

CSF Production

The primary function of the choroid plexus is to generate cerebral spinal fluid. As recently as 2007, it was believed that the choroid plexus epithelium created up to 70% of the cerebral spinal fluid production (Welch et al., 1966; Praetorius, 2007). However it is now thought to produce approximately 80% of the CSF, leaving the other 20% originating from interstitial fluid created by the blood brain barrier (BBB) (Damkier et al., 2013). CSF is generated at the rate of approximately 400 mL per day for a human (Johanson et al., 2005). The actual volume of CSF at any given moment in a human is approximately 140 ml (Pardridge, 2011). For comparison, the volume of CSF in dog is 7.8-24 mL (Artru, 1984), 1.4 – 2.3 mL in rabbit (McComb et al., 1982), 280-300 uL in rat and 40 uL in mouse (Artru, 1994). It is important to note that CSF is not simply plasma ultrafiltrate (Praetorius, 2007). Ninety-nine percent of the CSF is comprised of water with the other 1% containing ions (Na^+, K^+, Mg^+, Cl^-, HCO_3^-) and small proteins such as albumin, IgG, and trasferrin (Artru, 1999). The primary function of CSF is to provide buoyancy to support the weight of the brain itself, and also acts to provide protection against mechanical shock.

CSF Secretion from Choroid Plexus

Much literature exists on pressure-volume relationships of CSF in regards to hydrocephalus disorders and corresponding animal models (McMullen et al., 2012), yet the secretory processes and the transport systems involved in the secretion of CSF reamains under debate (Damkier et al., 2013). There is a growing consensus, however, that CSF secretion process is primarily due to an "isotonic" transport (Damkier et al., 2013) that utilizes a combination of aquaporins (AQP-1) (Oshio et al., 2003; Oshio et al., 2005) and ATP-ase ion channels such as sodium, chloride, potassium, and bicarbonate (Praetorius, 2007; Damkier et al., 2013) that create transcellular movement across the epithelium cells and into the ventricles.

CSF Circulation

The second purpose of CSF is to provide a means for the brain to eliminate waste products and also to provide a way for nutrients, hormones and immune cells to circulate in the CNS or to be delivered/removed from the CNS (Emerich et al., 2005; Praetorius, 2007). All of the aforementioned processes is governed by the CSF flow or circulation. The net movement or circulation of CSF along the spinal cord is driven by pulsations from cardiovascular heart and blood vessels. As CSF is secreted by the lateral ventricles it is drained into the third ventricle, continues via the cerebral aqueduct into the fourth ventricle, and then into the subarachnoid space of the spinal cord. During the contraction phase of the heart cycle (systole) pressure in the arteries of the brain increases. The increase in blood pressure drives CSF out of the brain through the upper cervical spine

because as blood volume rises CSF volume must decrease. During the relaxation phase (diastole) the pressure drops and CSF enters the cranial vault through the subarachnoid space of the upper cervical spine.

Respiratory pressure changes supply a steady rhythm of pressure such that, as the chest cavity drops during inhalation, due to the diaphragm moving down and the chest wall moving out, CSF is pulled out of the cranial vault. As pressure in the chest cavity increases during exhalation CSF is driven into the cranial cavity. Thus, combined cardiorespiratory waves are most important to the circulation of CSF through the brain and cord (Praetorius, 2007; Damkier et al., 2013).

The Choroid Plexus As a Target for Gene Transfer

The choroid plexus secretes bioactive peptides and has been considered as a potential target tissue for gene targeting (Gonzalez et al., 2010; Gonzalez et al., 2011). Targeting the central nervous system through traditional drug delivery methods (i.e. oral, intra-muscular, intravenous, etc.) has been limited due to challenges in circumventing blood-brain barrier (BBB). Accessing the cerebral spinal fluid via direct intrathecal (i.t.) or intracerebral ventricular (i.c.v.) injection (both of which bypass the BBB) can provide access to the CNS and to the choroid plexuses.

Intrathecal delivery of compounds via direct lumbar puncture (Hylden and Wilcox, 1980) enables small molecules or larger macromoleculesto distribute to the spinal cord parenchyma, and to the dorsal root ganglia sensory neurons. There is also rostral distribution to higher brain regions by both small molecules (Hylden and Wilcox,

1980) and AAV viral vectors (Schuster et al., 2014a; Schuster et al., 2014b). The combination of intrathecal delivery and AAV-mediated gene transfer utilizes the CSF in the subarachnoid space to reach the apical side (CSF side) of the choroid plexus. This makes the choroid plexus an accessible target for the AAV-mediated gene transfer following intrathecal deliver.

We have previously reported that *in vivo* intrathecal delivery (direct lumbar puncture) of AAV5 carrying the gene for green fluorescent protein (GFP) results in transduction of the choroid plexus in the 4th ventricle and to a lesser degree in the 3rd and lateral ventricles of mice (Schuster et al., 2014a; Schuster et al., 2014b). These studies were conducted in the presence of an i.v. mannitol (25%) pre-treatment, which is known to enhance penetration of the spinal cord and brain parenchyma. We have since demonstrated that in the absence of mannitol, intrathecal injection of AAV5-GFP transduces the choroid plexus without appreciable concomitant penetration of the brain parenchyma, AAV8-GFP does not transduce brain parenchyma and AAV9-GFP transduces a significant amount of the choroid plexus epithelium and also surrounding brain parenchyma (Schuster et al., 2014b). In order to further consider the utility of targeting the choroid plexus using viral gene vectors, we evaluated the ability of AAV5, AAV8 and AAV9 to transduce choroid plexus explants *in vitro* carrying the gene for green fluorescent protein (GFP).

As stated earlier, the objective of the present study was to evaluate gene transfer to the epithelial cells of the choroid plexus. To accomplish this objective we used several strategies. To directly compare gene transfer *in vitro* to the *in vivo* anatomy studies, we

elected to use green fluorescent protein (GFP) as the marker for gene transfer. We also chose to compare AAV5, AAV8, and AAV9 AAV serotypes as delivery vectors since they are commonly and increasingly used to target the CNS (Daya and Berns, 2008; Zincarelli et al., 2008; Beutler and Reinhardt, 2009; Hester et al., 2009; Van Vliet et al., 2009; Mason et al., 2010; Schuster et al., 2013; Dirren et al., 2014).

In order to facilitate future *in vitro* mechanistic and physiological studies of gene transfer to the choroid plexus we pursued establishment of choroid plexus *in vitro* systems in three ways. First, we attempted to establish primary rat choroid plexus cell cultures from dissociated epithelial cells harvested from freshly dissected rodent choroid plexus (Villalobos et al., 1997; Zheng et al., 1998). Second, we pursued establishment of organotypic *in vitro* choroid plexus explants according the method of Gonzalez et al. (2010). Finally, we acquired an immortalized choroid plexus cell line, developed by a Purdue University faculty member (Zheng and Zhao, 2002) and widely used in studies of blood-choroid plexus barrier transport (Szmydynger-Chodobska et al., 2007; Kläs et al., 2010). Through these studies I established both proof-of-concept that the choroid plexus epithelial cells are transducible by all three serotypes and the relative time course for gene transfer *in vitro* by each of these serotypes.

MATERIALS AND METHODS

Primary Choroid Cultures: Dissociated Cells

Male ICR-CD1 mice (Harlan, WI), 21-24 grams were used for dissociated *in vitro* experiment. Subjects were housed in groups of four in a temperature- and humidity-controlled environment and maintained on a 12-hour light/dark cycle with free access to food and water. These experiments were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

All cell culturing solutions and reagents are from Gibco® by Life Technologies (Grand Island, NY), unless otherwise noted. Fourth ventricle choroid plexus tissue was extracted from twenty mice and dissociated to cells according to the method of Zheng and colleagues (1998). Cells were counted with a hemacytometer using the trypan blue (0.4%) (Sigma) exclusion method for cell viability. Twenty choroid plexuses derived from the 4^{th} ventricle yielded approximately 100,000 cells per mL. Cells were seeded 50,000 cells per well in 24-well plate with glass coverslips pre-coated with laminin. The media contained the following: 50% DMEM, 50% F-12 Media with 5% FBS (Hyclone), 1% 5fU, 1% antibiotic-antimycotic, and 0.01% EGF (10 ng/ml). Half of the wells were treated the same day with 5 x 10^{10} vg per well of AAV5-GFP and returned to the incubator at 95% O2 and 5% CO2. The day of fixing, the coverslips were treated with a solution of 4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer, pH 6.9. The coverslips were rinsed and then incubated at room temperature for 1 hour in diluent (PBS containing 0.3% Triton, 1% BSA, and 1% normal donkey serum) and incubated overnight at 4°C in primary antisera diluted in the same solution. Primary antibodies

used; Rb anti-GFP 1:1000 (Invitrogen), Rb anti-TTR 1:1000 (Dako, Carpinteria, CA). After rinsing with PBS, sections were incubated for 1 h at room temperature with appropriate combinations of Cy2- and Cy3-, (1:300) conjugated secondary antisera (Jackson ImmunoResearch, West Grove, CA).

Primary Choroid Explants

Subjects were Sprague-Dawley male rats (Harlan, Madison, WI) with an average weight 200g. Subjects were housed in groups of four in a temperature- and humidity-controlled environment and maintained on a 12 hr light/dark cycle with free access to food and water. Subjects were anesthetized by isoflurane-overdose and the brain was carefully removed intact. Fourth ventricle choroid plexus was removed by lifting the cerebellum. Third and lateral ventricles were exposed by making a parallel incision approximately 5 mm from the midline. Forceps were used to extract the choroid tissues and placed in media described above. The procedures for culturing, incubation, fixation and immunohistochemistry were the same as described in section above.

Immortalized Choroid Plexus Cell Line (Z310 Cells)

All cell culturing solutions and reagents are from Gibco® by Life Technologies (Grand Island, NY), unless otherwise noted. The Z310 cell line, a cell line reported to be representative of the choroid plexus (Zheng and Zhao, 2002), was a generous gift of Prof. Wei Zheng of Purdue University and was obtained on the Material Transfer Agreement on file with Office of Sponsored Projects Administration at Purdue University and the

University of Minnesota. The cells arrived on dry ice and were immediately transferred to flask culture as per enclosed Zheng Lab standard operating procedure. An 85-90% confluent flask yielded on average 7 - 8 x 10^6 viable cells per mL via hemocytometer and trypan blue assay (0.4%, Sigma Aldrich). *In vitro* cell cultures of the immortalized choroid epithelium Z310, were plated at a ratio of 1:64 in a media consisting of 10% FBS, 1% pen/strep, 0.2% gentamicin, 0.01 hEGF in sterile high glucose DMEM. The growth of the Z310 cells is very robust and reached confluency in 24-36 hours post seeding. Therefore a slight modification was made by using low glucose DMEM to slightly inhibit the growth of the cells.

For immunohistochemical staining, fixation was performed by using a 4% paraformaldehyde solution. These fixed coverslips were rinsed by gentle dipping into room temperature PBS. Primary antibodies used were chicken anti-GFP (1:2000) (Abcam™) with a secondary of Cy-2 donkey anti-chicken (1:300) (Jackson ImmunoResearch, West Grove, CA). The coverslips were again rinsed by gentle dipping in room temperature PBS and were mounted to slides with Vectashield with DAPI mounting medium (Vector Laboratories, Burlingame, CA).

Well Plate and Cover Slip Preparation

Prior to seeding, 22 mm x 22 mm coverslips, No. 1 thickness (Erie Scientific Company, Portsmouth, NH) were washed in 0.1M HCl at a temperature of 50-60°C for 1 hour. Triple rinsed with DI water and triple rinsed with 95% ethanol and stored in 95% Ethanol. The day of seeding, one coverslip per well of a sterile 6-well plate (Corning

3516, Corning, NY) was placed in each well and allowed to evaporate in culturing hood. A sterile solution of laminin (dissociated and explants cultures) or poly-D-Lysine (Z310 cultures) was applied to the coverslip within the well as per manufacturer protocol, and allowed to dry in the cell culture hood until dry.

Viral Vector

The viral vectors for adeno associated viral (AAV) vector serotype 5, 8, 9 (AAV5, AAV8, AAV9) were purchased from the University of Florida Vector Core (Gainesville, Florida). These vectors all contained the CMV promoter which is the same promoter used in the AAV-hADC-GFP construct. In all studies, each well was treated with 2.5 μL vector stock/well of the following: AAV5 (stock concentration $7.17x10^{12}$ vg/ml), AAV8 (stock concentration 4.0×10^{12} vg/ml), or AAV9 (stock concentration 3.3×10^{11} vg/ml) or saline.

Immunopositive GFP Cell Counting (Blinded)

The degree and time course of transduction was assessed with immunohistological staining methods using antibodies against and GFP (Abcam ™). The numbers of positive GFP cells were quantified by two separate observers who were blinded to the treatment and time points. Briefly, the slide was placed on the microscope and viewed under the UV channel at 40X magnification. An area was randomly selected and the DAPI labeled nuclei were brought into focus. Once in focus, the channel was switched to Cy-2 to observe GFP positive cells. If present, the number of GFP positive

cells and the relative area was noted. The observer then moved to another location and repeated the process for up to 10 locations on the 22 mm x 22 mm coverslip and recorded their counts in the laboratory notebook. No significant difference was found between the values obtained by the two blinded experimenters (Student's t-test, $p<0.05$).

RT-PCR

Total RNA was extracted from Z310 cells treated with AAV9-hADC-GFP, and RT-PCR was performed using the techniques described in Chapter 2.

RESULTS

Primary Choroid Cultures: Dissociated Cells

In the dissociated mouse choroid experiments it was confirmed that the cells exhibited presence of transthyretin (TTR). Transthyretin is secreted by the choroid plexus into the cerebrospinal fluid and is used as a marker of choroid plexus epithelial cells (Monnot and Zheng, 2013).

Green fluorescent protein (GFP) was detected immunohistochemically in dissociated choroid plexus epithelial cells after 48 hours in culture and co-localized in cells expressing transthyretin (Figure 4.1C and 4.1D). Such co-localization confirms AAV5-GFP gene transfer to choroid plexus epithelial cells. However, the number of cells transduced was quite limited. We reasoned that a longer incubation time might be needed. However, it was noted that there was pronounced lifting of the cells from the laminin pre-treated cover-slips at 2 days in culture and therefore, our assessment was that the acute dissociated culture approach would have limited utility for time-expression studies of gene transfer to choroid plexus epithelial cells.

Primary Choroid Explants

It was next attempted to optimize a system using explants of intact choroid plexus from the fourth ventricle. The rationale was that maintaining the structural architecture (microvasculature included) of the choroid plexus tissue may better mimic the *in vivo* condition. With dissociated cells, the combination of 20 fourth-ventricle choroid plexuses yielded only enough cells to seed 4 wells, enough for 2 treated wells and 2 control wells. It was suggested that plating each choroid separately, but intact, would result in improved sample survival. This approach enabled additional vectors to be tested as well as reduce the need for media changes.

The experiments treating intact choroid plexuses of rat allowed for a total of 24 choroid explants to be plated which enabled a viral particle concentration-response study for both AAV5-GFP and AAV8-GFP to be conducted. The explants were either treated with 1 μL of PBS (n=6), 1X (10^9 vp/well), 0.1X (10^8 vp/well) or 0.01X (10^7 vp/well) dilutions of the AAV5-GFP or AAV8-GFP (n = 3 per treatment group per vector). Compared to the dissociated culture approach, the explant technique facilitated a longer post-viral vector incubation time; the choroid plexuses were fixed after 72 hours post-AAV-GFP treatment. Upon viewing with fluorescence microcopy, it was determined that the explants receiving the 1X treatment (10^9 vp/well) of AAV5-GFP or AAV-8-GFP resulted in the strongest GFP visualization (Figure 4.2, lower concentrations not shown).

Control explants did not result in GFP staining (Figure 4.2A). In the treatment explants, the AAV5-GFP-treated choroid plexus showed greater immunopositivity for

GFP, although those treated with AAV8-GFP were also positive for GFP relative to control. Three of the controls were not fixed but stained with trypan blue and assessed for viability. The cells on the outside surface took up trypan blue indicating that the cells were dead. However, the center of the explants excluded trypan blue, even after 30 minutes of incubation. It was noticed that the vascularization of the choroid explants was visible under 20X magnification 72 hours after extracting. This vasculature was not visible at 20X magnification immediately after extraction. It was noted that microscopy on the 3-dimensional explant proved difficult to achieve one plane of focus and images were taken on the periphery of the tissue under the coverslip. Overall, the GFP expression observed in the choroid plexus explants concurred with the dissociated cells in that the choroid plexus epithelial cells are amenable to gene transfer. However, here again, the limitations of survival of the total explant and difficulty of imaging with 3-D nature of sample, suggested that the system might be of limited utility for the studies planned.

Immortalized Choroid Plexus Cell Line (Z310 Cells)

In the course of developing and researching other choroid cell culturing techniques it came to our attention that the Z310 immortalized mouse choroid plexus epithelial cell line might be beneficial to the proposed studies. By using low glucose media instead of high glucose DMEM the immortalized cells allowed for incubation times up to 96 hours without the need to change media, enabling a significantly greater duration of exposure to the viral particles. The wells were treated with 2.5 μL of either saline AAV5-GFP, AAV8-GFP or AAV9-GFP. The increased treatment volume was to adjust for the use of larger 6-well plates instead of 24-well plates. After 24 hours, cells

immunopositive for GFP were present (Figure 4.3) in all AAV-treated cells. This pattern continued and increased for each vector and each subsequent time point (Figure 4.3).

After fixation and immunohistological labeling, it was noted at 48 hours that the cell layer was confluent on the coverslip. By 72 hours the cell layer was proliferating vertically and appeared 2-3 layers thick in multiple areas on the coverslip. By 96 hours the cell layer was 4-5 layers thick. Therefore, the images were taken in slices and compiled to produce a planar image.

There was a significant difference in GFP expression between 24 and 96 hours in AAV5-, AAV8-, and AAV9-GFP-treated groups as determined by one-way ANOVA; this observation documented an overall effect of time on GFP expression (Figure 4.4). Tukey's post-hoc test for multiple comparisons between groups confirmed significant difference between most time points. In summary, the results of this time course study indicate that AAV5, 8, and 9, were all capable of transducing the immortalized choroid plexus epithelial cells in a time-dependent manner. The robustness of the system led us to conclude that the Z310 cell line offers the greatest utility for studies of gene transfer to the choroid plexus.

Gene Transfer of AAV5-hADC to Immortalized Choroid Plexus Cells

Following optimization of the culturing parameters for the Z310 cells a small study was performed in which Z310 cells were exposed to AAV5-hADC-GFP. The cells were treated with with 2.5 μL of PBS or AAV9-hADC-GFP. After 72 hours, the cells were collected and a portion of them were analyzed for hADC mRNA content. The result indicated that the Z310 cells do not have native hADC present but the cells treated with

AAV5-hADC-GFP were positive for hADC mRNA content. These results confirmed that the Z310 cells are amenable to AAV5-mediated gene transfer and can express mRNA from a second gene; this cell line may prove optimal for further studies of hADC expression and possible release of agmatine from the choroid plexus.

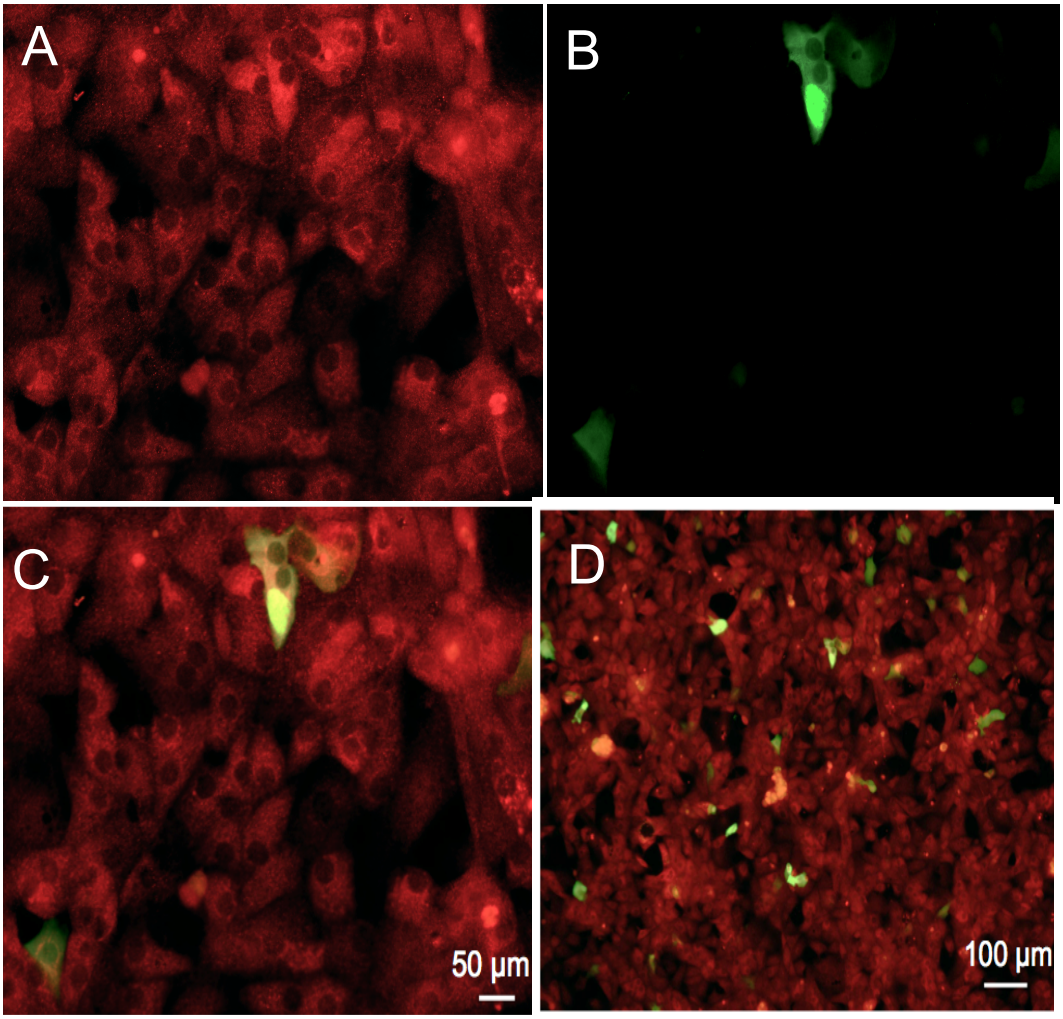

Figure 4.1: AAV *in vitro* delivery to dissociated primary mouse choroid culture 48 hours post treatment. A) Red = Transthyretin (TTR) (choroid cell marker) B) Green= GFP; Yellow indicates co-localization at C) 40X and D) 10X magnification.

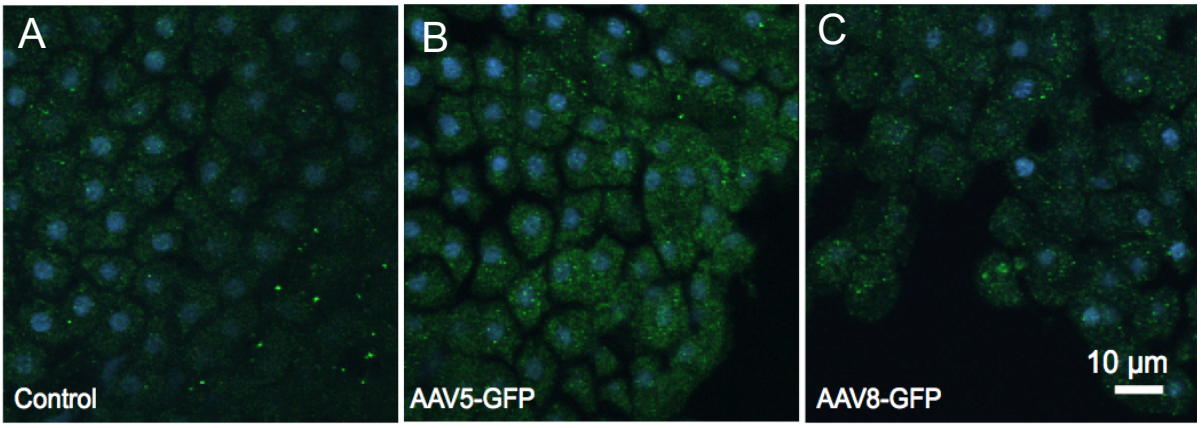

Figure 4.2: AAV5-GFP and AAV8-GFP *ex vivo* delivery to intact rat choroid plexus explants at 60X. A) Untreated control B) AAV5-GFP treated *ex vivo*. C) AAV8-GFP treated *ex vivo*. (Green = GFP, Blue = DAPI)

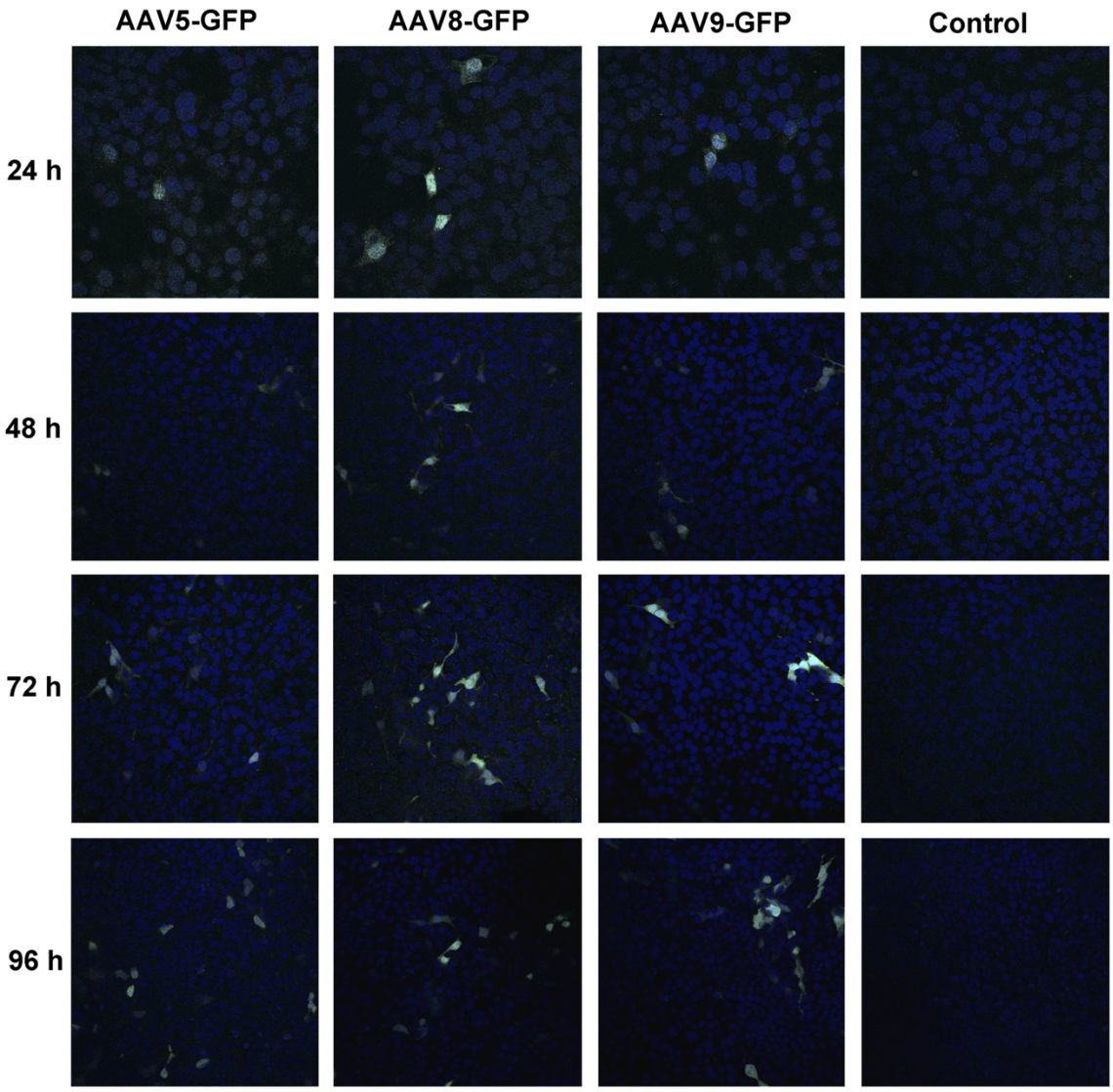

Figure 4.3: AAV5-GFP, AAV8-GFP, and AAV9-GFP delivery to immortalized choroid plexus cell line, Z310, 24, 28, 72 and 96 hours post treatment. (Green = GFP, Blue = DAPI, White = Colocalization)

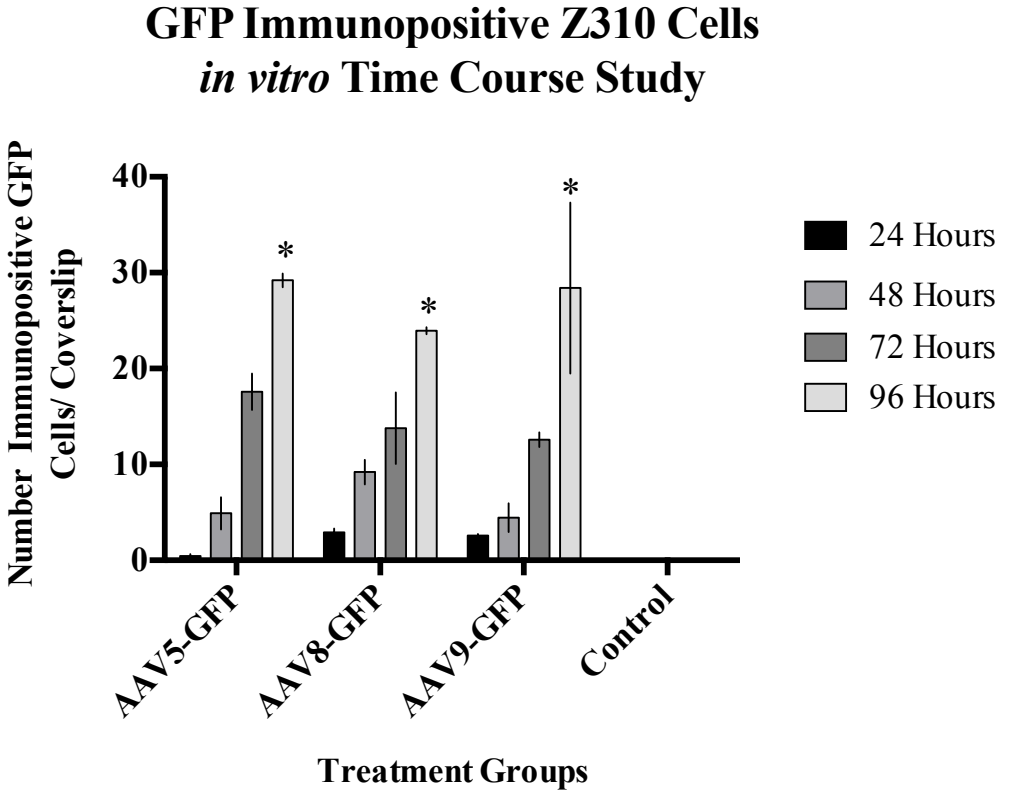

Figure 4.4: Bar graph representation of positive GFP cells in Z310 cells over time: 24-, 48-, 72-, and 96-hours post treatment with either AAV5-GFP, AAV8-GFP, AAV9-GFP or saline. * Indicates a significant difference between 24 hours and 96 hours within each treatment group, AAV5-GFP ($p<0.0001$), AAV8-GFP ($p<0.0005$), and AAV9-GFP ($p<0.0001$) One-way ANOVA with Tukey's post hoc test for multiple comparisons between groups.

DISCUSSION

Despite decades of sophisticated targeting of a wide variety of molecular systems identified as important for chronic pain, management of chronic pain remains an area in grave need of new approaches. Gene transfer utilizing the non-pathogenic adeno-associated virus shows great promise in the specific targeting of CNS tissues, especially those related to chronic pain (Beutler and Reinhardt, 2009; Vulchanova et al., 2010). By utilizing the cerebral spinal fluid (CSF) in the subarachnoid space, the viral vector particles can be distributed to the brain thereby transferring long-term genetic modifications to the choroid epithelium. Specifically, AAV is a non-pathogenic virus with multiple serotypes that have unique tropisms to a variety of tissues. In order to further characterize AAV gene transfer to the choroid plexus we have applied an *in vitro* and *ex vivo* method for culturing primary and intact choroid tissue collected from both mouse and rat respectively. We show here that both AAV5 and AAV8 as well as AAV9 target the choroid plexus *in vitro* and *ex vivo*.

SUMMARY AND CONCLUSIONS

Although the choroid plexus is not a tissue commonly associated with sensory processing, we have shown previously in *in vivo* studies that intrathecal delivery of specific AAV vectors will transduce choroid plexus epithelial cells (Schuster et al., 2014a; Schuster et al., 2014b). Based on this previous *in vivo* experimentation, we originally predicted that AAV5 and AAV9 would yield greater transduction and tropism to choroid plexus epithelium relative to AAV8. Through the present study in three

distinct *in vitro* preparations we have confirmed the amenability of choroid plexus epithelial cells to AAV-mediated gene transfer, by all three AAV serotypes. These initial studies have provided information on optimal *in vitro* choroid plexus cell culture conditions for future studies of in vitro gene transfer.

The results presented here in Chapter 4 illustrate the utility of the *in vitro* immortalized choroid plexus cell line method for further evaluation of gene expression and function in choroid plexus epithelial cells. Gene transfer to the choroid plexus following intrathecally delivering adeno-associated viral gene therapy may represent potentially advantageous strategy for the delivery of gene therapy for neurological disorders, especially that of chronic pain.

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Chapter V. *Summary, Future Directions, and Conclusion.*

SUMMARY

The research approach and observations featured in this thesis have shown the feasibility of gene transfer *in vivo* (Chapter 2) and *in vitro* (Chapter 4) to central nervous system related-tissues or choroid plexus cells, with AAV viral vectors. The quantification results in Chapter 3 suggest that the observed increase in spinal concentrations of agmatine is due to the expression of hADC enzyme in transduced regions of the CNS. The observation of elevated spinal agmatine presented in Chapter 3 corroborates the findings of Chapter 2 where subjects treated with AAV-hADC fail to develop opioid analgesic tolerance. Together those observations parallel that of previous observations showing that spinally delivered exogenous agmatine prevents the development of both morphine (Fairbanks et al., 1997) and endomorphin-2 analgesic tolerance (Wade et al., 2009). Given the significant challenge in chronic use of opioids for the clinical management of chronic pain, the observation that expression of human ADC prevents the development of opioid analgesic tolerance suggests that this strategy could be clinically advantageous as a gene therapy. However, the source of the elevated agmatine and potential long-term effects are not yet fully understood.

FUTURE DIRECTIONS

The data presented in this thesis demonstrate that gene transfer to the choroid plexus *in vivo* results in elevated expression of hADC. Expression of hADC in choroid plexus may result in the ability of freely available L-arginine to be converted into agmatine. It is possible that the elevated agmatine is then secreted from the epithelial choroid plexus cells and circulated through the CSF. The human choroid plexus epithelial cells exhibit near identical distribution of transporters to the of rat and mouse choroid plexus (Damkier et al., 2013). Therefore, there should be good correspondence between the pre-clinical models and the human condition. However, there was some notable differential transduction of choroid plexus between *in vivo* and *in vitro* systems. We suspect this is due to differences in the cell surface receptors of the choroid plexus epithelial cells or diffusional differences in the *in vivo* environment. This may limit the applicability of the *in vitro* system with respect to some viral vector serotypes.

As for the utility of the Z310 immortalized cell line, it has been proposed that the Z310 cell lines are similar to *in vivo* choroid plexus in regards to transthreytin production and polarity (Zheng 2002, Kitazawa et al., 2001; Ramanathan, 1996; Zheng, 1998), as well as the functional transporters P-gp and Mrp1 (Kläs and Reichel, 2010). However, a recent study has raised issue when using this cell line as a true representative of the blood-cerebrospinal fluid barrier (BCSFB) due to lack of presence of certain tight junction proteins (Kläs and Reichel, 2010). For our studies of gene expression the latter limitation may not be of significant concern. The *in vitro* choroid plexus epithelial cells system that we characterized in Chapter 4 would enable studies to further characterize the

outcomes of AAV gene transfer to choroid plexus and subsequent release of therapeutics into the CSF.

The potential therapeutic utility of selective gene targeting to the choroid plexus has been recognized by others (Gonzalez et al., 2010; Gonzalez et al., 2011; Haddad et al., 2013); however, to our knowledge, methods to exclusively transduce the epithelial cells of the choroid plexus remain limited at the present time. Rejev and colleagues (2010) reported that the choroid-specific promoter corticotropin releasing factor receptor type 2 beta (CRFR2β) provides exquisite specificity for the choroid plexus. Lentiviral vectors containing this promoter and the gene for GFP demonstrated expression of GFP only in choroid epithelial cells following intracerebroventricular (i.c.v.) delivery. In another study, it was found that the baculovirus specifically target the choroid plexus when delivered i.c.v. (Lehtolainen et al., 2002).

A second strategy is currently being developed by Kaler and colleagues at the National Institutes of Health. This research group is reportedly undertaking the selective development of a recombinant AAV vector with specific viral capsid motifs that will specifically target choroid plexus cells (Kaler, NIH). Application of either of these choroid-specific molecular tools would importantly enable true assessment of the utility of choroid plexus-specific gene targeting for a broad spectrum of CNS disorders.

The impact on the intrinsic functions of the choroid itself *in vivo* (such as changes in CSF production) when AAV gene therapy is administered would also need to be assessed. It would be essential that CSF production proceeds unchanged (McMullen et al., 2012). Some initial studies have evaluated CSF pressures and ventricle size following

viral vector delivery (McMullen et al., 2012). These types of long range toxicity studies would need to be conducted extensively to assess safety of AAV-mediated gene transfer to the choroid plexus (Beutler and Reinhardt, 2009; Kumar et al., 2011).

Even though it is known that AAV vectors can be modified in such a way as to provide therapeutic or beneficial treatments in pre-clinical models, the translation of such therapy to humans has been proceeding with "cautious optimism" (Asokan et al., 2012). This cautiousness is due to unpredicted, adverse, immunological reactions to viral-mediated gene therapies. During the advent of gene therapy there was much excitement over the possibilities and future of gene therapy. The successful treatment of a young child that was cured of her X-linked Severe Combined Immune Deficiency (Blaese et al., 1995) provided motivation for researchers to explore other therapies. However, with time, other children receiving a similar therapy developed leukemia due to the mutagenesis of the virus integration near a proto-oncogene (Hargreaves, 2002) and the trials have since been stopped. There have also been tragic fatalities that have been widely covered by the media. One well known case is the treatment of Jesse Gelsinger, an eighteen year old who died after receiving gene therapy to correct his ornithine transcarbamylase deficiency (Raper et al., 2003). These cases brought to light many considerations when using gene transfer as a therapy, such as the fact that there is no *off switch* in gene-transfer therapy and that the immunological responses of every individual cannot always be accurately predicted (Kimmelman, 2009). Thus far, pre-clinical data has reported little to no adverse side effects with centrally delivered AAV-mediated therapies (Beutler et al., 2005; Hadaczek et al., 2010; Samaranch et al., 2012; San

Sebastian et al., 2013); however there is no guarantee the same will hold true in humans. Therefore the FDA has worked with researchers and clinicians to create a guidance for gene therapy clinical trials (FDA, 2006) that call for extensive toxicological studies and long-term immunological monitoring of subjects immune status many years following gene delivery (FDA, 2006; Kimmelman, 2009). However, these guidances are often written for studies utilizing viral vectors with a high chance of reactivation or delayed adverse events, such as gammaretrovirus, lentivirus, and herpes virus (FDA, 2006). How AAV-mediated gene transfer fit into these guidances, is not specifically addressed.

In his book chapter, describing the risks of gene transfer in clinical research, Kimmelman emphasizes that there is a specific need for uniform regulations and systematic testing of AAV-mediated therapies (2009). There is also a need for standardization, not only for study design, but for a system that collects safety and toxicological data in an organized and easily accessible fashion (Kimmelman, 2009). As this relates to the work in this thesis, more research is necessary to assess the long-term safety of i.t. delivery of AAV-hADC in mice and eventually larger animal models and potentially non-human primates. Others have taken this approach and are finding in Phase I trials that AAV delivery is tolerable when administered directly into the CNS (Beutler et al., 2005; Hadaczek et al., 2010; Samaranch et al., 2012; San Sebastian et al., 2013).

There is a general consensus that AAV is nonpathogenic and is naturally replication defective. There was a reported case of hepatotoxicity due to the AAV2 serotype vector in a therapy for hemophilia (Manno et al., 2006). Since most adults are

seropositive for AAV antibodies, delivery of AAV-gene transfer is often unsuccessful with intravenous or intramuscular administration (Chirmule et al., 1999). The AAV capsid is the primary interface between the host and vector genome and it is believed that the capsid moderates specificity of transduction and immune response. Second generation AAV vectors have been re-engineered to lessen the immune response to the AAV capsid (for a full review see Asokan, et al., (2012). AAV vectors have a variety of serotypes to enable cell targeted entry (Gao et al., 2005), and display unique tissue tropisms. Thus, AAV-mediated gene transfer has become a hopeful strategy in the translation of animal-based research to human clinical trials (Beutler and Reinhardt, 2009; Asokan et al., 2012).

CONCLUSION

As stated in the Introduction to this thesis, the National Institute of Medicine estimates that over 116 million Americans have common chronic pain conditions (Institute of Medicine Committee on Advancing Pain Research, 2011). Yet, the most current and popular treatments often fail to achieve all the therapeutic goals for eliminating or significantly reducing the patient's discomfort. Often times the patient's treatment will be temporary and can lead to invasive procedures or escalated doses of pharmaceutics associated with unacceptable side effects (Finegold et al., 1999). The financial ramifications of chronic pain are severe with costs estimating $635 billion or more each year in medical treatments and lost productivity (Institute of Medicine Committee on Advancing Pain Research, 2011). Therefore, new approaches and

solutions to chronic pain are necessary such as the gene therapies described in this thesis. It is hoped that the information gathered as a result of this doctoral thesis research project will assist in the goal of discovering new approaches to treating and ameliorating chronic pain.

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