GLT-1 OVER-EXPRESSION ATTENUATES VISCERAL NOCICEPTION BY PHARMACOLOGICAL AND GENE THERAPY APPROACHES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Kenny M. Roman, B.S.
Graduate Program in Integrated Biomedical Science Program

The Ohio State University
2012

Dissertation Committee:
Dr. Robert L. Stephens Jr., Advisor
Dr. D. Michele Basso
Dr. C.A. Tony Buffington
Dr. Joseph B. Travers
Abstract

The underlying physiopathology of chronic visceral pain conditions remains unclear due to the complexity of visceral nociceptive signaling. Therefore, therapeutic pain management is suboptimal. Afferent neurons utilize glutamate as the principle neurotransmitter to signal nociceptive activity from visceral organs. Previous studies suggest that transgenic or pharmacologic approaches to over-express the predominant glutamate transporter, GLT-1, responsible for removal of glutamate, produce significant reduction in visceromotor response to colon distension. Thus, strategies to decrease glutamate availability in the synaptic cleft may be useful for visceral pain management.

This dissertation explored the effect of GLT-1 over-expression on visceral nociception caused by bladder irritation/inflammation, cross-organ sensitization, and neonatal stress. Moreover, this dissertation addresses the site of action of the anti-nociceptive effect of GLT-1 over-expression, the mechanism of action involving glutamate receptor trafficking, and the efficacy of GLT-1 over-expression long-term.

Studies began by examining differences in glutamate uptake and GLT-1 expression in mice treated with ceftriaxone (CTX) for seven days and showed a significant increase in GLT-1 expression and activity compared to control. Next, it was demonstrated that GLT-1 over-expression attenuated the visceromotor response (VMR) to urinary bladder distension (UBD). Moreover, irritation or inflammation of the bladder, which enhances the nociceptive response to UBD, was attenuated by 1 week (1-wk) CTX
treatment. The next study addressed the site of action of GLT-1 over-expression to mitigate visceral nociception. Intrathecal treatment with dihydrokainate, a selective GLT-1 antagonist, 1 hour before bladder distension and revealed a dose-dependent reversal of the CTX-mitigated visceromotor response to urinary bladder distension. Moreover, the anti-nociceptive effect of CTX induced GLT-1 over-expression was diminished after 30 days in mice.

Finally it was shown that adeno-associated virus serotype 9 (AAV9) 1) crossed the blood-brain barrier and transduced the parenchyma following intravascular neonatal gene delivery, 2) AAV9 carrying the EAAT2 (mouse homologue GLT-1) protein gene increased the glutamate uptake activity, and 3) the AAV9-EAAT2 construct attenuated the nociceptive response to colon distension at the highest distension pressure (60 mm Hg) in adult animals. In summary, these exciting findings suggest that GLT-1/EAAAt2 over-expression represents a mechanistic-based approach to mitigate visceral pain.
Dedication

This dissertation work is dedicated to my family.
Acknowledgments

The time I have spent working to complete this dissertation has brought me immense joy and taught me valuable lessons that I am certain will help me throughout my life and career. I’ve had the pleasure to meet people that have kept me on the path to success and their great advice has allowed me to continue after difficult moments. Hence, I would like to thank the people that have made this dissertation possible:

I am immensely grateful for the mentorship of my advisor Dr. Robert L. Stephens, Jr. who has showed constant support and provided me the venue to fulfill my career goals. I appreciated Dr. Stephens helpful advice, since it has always led me to grow as a researcher and kept me focused on my goals. As a result, I have learned valuable lessons that will prove crucial in my scientific career.

I would like to thank my dissertation committee; Dr. Joseph B. Travers, Dr. D. Michele Basso, and Dr. A.C. Tony Buffington for their unwavering support, valuable insight of my dissertation work, and generous offers to help me throughout the dissertation process. Also, I would also like to thank Dr. Jackie D. Wood, a general examination committee member for his encouragement and showing interest in my work. Moreover, I would like to thank Dr. Virginia M. Sanders for her kindness, guidance, and assistance. A special thank you to Dr. Allan J. Yates for giving me the opportunity to fulfill my career goals by accepting me to the IBGP program.
Next, I would like to acknowledge people who have collaborated in my dissertation projects and have been instrumental for designing experiments. Dr. Brian K. Kaspar and Dr. Kevin D. Foust thank you for your collaboration, both, played an important part in my development as a researcher and provided the scientific tools to carry my dissertation work forward. Dr. Howard H. Gu thank you for allowing me access to your lab and helpful input on glutamate uptake assays.

Moreover, I will always be grateful to my dear friends Dr. Arthur J. Pope and Dr. Rodrigo M. Velarde for their advice, support, encouragement, and help throughout my graduate school experience. Furthermore, I would like thank Keerthi Thirtamara Rajam for his support and friendship. To former and current members of the lab: I would like to thank Dr. Yuan Lin for his companionship and several contributions to my dissertation projects and acknowledge Dr. Min Yang for her assistance and help with experimental designs. In addition, thank you Marie Corbo for your assistance with my dissertation work.

My dissertation work could not have been possible without the financial support from the NIH and Porter Fellowship from the American Physiological Society. Therefore, thank you for your investment and for helping me achieve my career goals. This dissertation is a symbol of my appreciation to friends, faculty, and institutions that contributed to my dissertation work.
Vita

2001-2006 ........................................... B.S., Chemistry/Molecular and Cellular Biology, University of Illinois at Urbana-Champaign

2006 to present...................................... Graduate Research Associate, Integrated Biomedical Science Program, The Ohio State University

2010 to present...................................... Porter Fellowship, The American Society of Physiology
Publications


(* contributed equally to work)

doi:10.1155/2011/507029

Field of Study

Major Field: Integrated Biomedical Science
Abstract................................................................................................................................................. ii

Dedication ............................................................................................................................................. iv

Acknowledgments ................................................................................................................................. v

Vita........................................................................................................................................................... vii

Publications ............................................................................................................................................ viii

List of Tables .......................................................................................................................................... xii

List of Figures ........................................................................................................................................ xiii

Symbols and Abbreviations ................................................................................................................... xv

Chapter 1 ............................................................................................................................................... 1

Introduction to Somatic and Visceral Pain .......................................................... 1

1.1 Pain ................................................................................................................................................. 1

1.2 Somatic Sensory System .................................................................................................................... 4

1.3 Visceral Sensory System .................................................................................................................... 6

1.4 Somatic vs. Visceral Pain..................................................................................................................... 8

1.5 Cross-organ sensitization .................................................................................................................. 13
1.6 Behavior Model .......................................................... 17
1.7 Chronic Visceral Pain ................................................... 19
1.8 Glutamate Receptors .................................................... 22
1.9 Glutamate Transporters .................................................. 27
1.10 GLT-1 role in pain ......................................................... 32
1.11 Problem Statement ....................................................... 35
1.12 Objectives and Research Questions ................................. 37
1.13 Significance of the Study ............................................... 38
1.14 Definition of Terms ...................................................... 38

Chapter 2 ........................................................................... 41
GLT-1 over-expression attenuates bladder nociception and local/cross-organ sensitization of bladder nociception ................................................................. 41

2.1 Introduction ..................................................................... 41
2.2 Methods ......................................................................... 43
2.3 Results ............................................................................ 50
2.4 Discussion ....................................................................... 63

Chapter 3 ........................................................................... 69
Characterization of anti-nociception effect of GLT-1 over-expression: site of action, GluR1 trafficking, and neonatal stress model ......................................................... 69
3.1 Introduction ........................................................................................................................................ 69

3.2 Methods ........................................................................................................................................... 72

3.3 Results ............................................................................................................................................ 79

3.4 Discussion ....................................................................................................................................... 87

Chapter 4 ............................................................................................................................................... 93

Adeno-associated virus serotype 9 mediated transduction of EAAT2 gene attenuates visceral nociception ................................................................................................................................. 93

4.1 Introduction ................................................................................................................................... 93

4.2 Methods ......................................................................................................................................... 96

4.3 Results .......................................................................................................................................... 101

4.4 Discussion ..................................................................................................................................... 106

Chapter 5 ............................................................................................................................................. 111

Conclusions and Perspectives ................................................................................................................. 111

5.1 Perspective ................................................................................................................................. 111

5.2 Conclusions and future directions ................................................................................................. 114

5.3 Final remarks .............................................................................................................................. 121

References ............................................................................................................................................ 123
List of Tables

Table 1.1 Human glutamate transporter nomenclature and rodent homologues. ............. 28
List of Figures

Figure 1.1 Somatic and visceral pain circuits ................................................................. 6
Figure 1.2. Visceral sensory pathways .................................................................................. 7
Figure 1.3. Arborization of the somatic and visceral afferents in the spinal cord. .......... 12
Figure 1.4. Cross-organ sensitization .................................................................................. 14
Figure 1.5. Dichotomizing afferents and autonomic-induced cross-organ sensitization. 15
Figure 1.6. Somatic and viscera sensory innervation to the spinal cord dorsal horn....... 17
Figure 1.7. NMDA receptor ................................................................................................. 24
Figure 1.8. Comparison of NMDA and AMPA activation during normal conditions and persistent inflammation ................................................................................................. 25
Figure 1.9. Glutamate transporters (EAAT1-3) found in astrocytes and neurons .......... 29
Figure 1.10. Glutamate transporter uptake ............................................................................. 30
Figure 1.11. Glutamate is converted to glutamine inside astrocytes .................................. 30
Figure 2.1. A-B. CTX induced GLT-1 over-expression and increased glutamate uptake. 51
Figure 2.2. A-B. Ceftriaxone treatment decreased VMR to UBD ....................................... 52
Figure 2.3. The effect of systemic injection of selective GLT-1 antagonist dihydrokainate (DHK) on GLT-1 over-expression ......................................................................................... 53
Figure 2.4. A-B. The effect of GLT-1 over-expression on bladder irritant-sensitized visceromotor response ................................................................. 55

Figure 2.5. Lower doses of CTX treatment do not produce a VMR different from the control ........................................................................................................... 56

Figure 2.6. Licking behavior increased after acrolein bladder infusion. .................... 58

Figure 2.7. High dose CTX treatment decreased VMR to UBD in mice with visceral hypersensitivity ............................................................................................... 59

Figure 2.8. Low dose CTX treatment decreased VMR to UBD in mice with visceral hypersensitivity ................................................................................................. 60

Figure 2.9. A-B. CTX treatment decreases TNBS-induced cross-organ sensitization..... 62

Figure 3.1. Dihyrokainate reverses the effect of ceftriaxone ................................. 80

Figure 3.2. A lower concentration of dihyrokainate reverses the effect of ceftriaxone... 81

Figure 3.3. Dihyrokainate at 0.003 mM does not reverse the effect of ceftriaxone ....... 82

Figure 3.4. AMPA subunit trafficking ...................................................................... 83

Figure 3.5. GluR1 trafficking is mitigated after GLT-1 over-expression...................... 84

Figure 3.6. GluR1 is increased in the membrane after cyclophosphamide treatment ..... 85

Figure 3.7. Neonatal stress induced visceral hypersensivity in adult mice ............... 86

Figure 3.8. Duration of CTX effect until day 30 .......................................................... 87

Figure 4.1. AAV9-GFP mediates transgene expression in the spinal cord................. 102

Figure 4.2. AAV9-EAAT2 induced glutamate transporter over-expression .............. 103

Figure 4.3. AAV9-EAAT2 enhanced glutamate uptake ............................................. 104

Figure 4.4. AAV9-EAAT2 decreased visceromotor response to CRD ..................... 105
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>ACRO</td>
<td>Acrolein</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CCI</td>
<td>Chronic constriction nerve injury</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRD</td>
<td>Colo-rectal distension</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>CTX</td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td>CYP</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>DCN</td>
<td>Dorsal column nuclei</td>
</tr>
<tr>
<td>DHK</td>
<td>Dihyrokainate Acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GLT-1</td>
<td>Glutamate transporter-1</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GluR</td>
<td>Glutamate receptor</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>IBD</td>
<td>Irritable bowel disorder</td>
</tr>
<tr>
<td>i.c.</td>
<td>intracolonic</td>
</tr>
<tr>
<td>IC/PBS</td>
<td>Interstitial cystitis/painful bladder syndrome</td>
</tr>
<tr>
<td>ICP</td>
<td>Intraluminal colonic pressure</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.t.</td>
<td>intrathecal</td>
</tr>
</tbody>
</table>

xvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ivc</td>
<td>Intravesicular</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MO</td>
<td>Mustard Oil</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B cells</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK-1</td>
<td>Neurokinin 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>P2X</td>
<td>Purinergic receptor</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal grey</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PSDC</td>
<td>Postsynaptic dorsal column</td>
</tr>
<tr>
<td>RVM</td>
<td>Rostral ventromedial medulla</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRPV</td>
<td>Transient receptor potential gene subfamily V</td>
</tr>
<tr>
<td>SNL</td>
<td>Spinal nerve ligation</td>
</tr>
<tr>
<td>UBD</td>
<td>Urinary bladder distension</td>
</tr>
<tr>
<td>VEH</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VMR</td>
<td>Visceromotor response</td>
</tr>
</tbody>
</table>

xvii
Chapter 1

Introduction to Somatic and Visceral Pain

1.1 Pain

The International Association for the Study of Pain (IASP) has defined pain as “an unpleasant sensory and emotional experience primarily associated with tissue damage (Merskey and Bogduk, 1994).” This definition implies a subjective component (cognitive and emotional input) to pain, which in humans involves extremely complex neuroanatomical substrates (Craig, 2003). Pain is an evolutionary preserved sensory system that serves as an early warning mechanism to protect tissue from harmful stimuli (Basbaum et al., 2009). Pain related reflexes across animal species are commonly used in research and defined as nociception. Animal models are useful in pre-clinical assessment of nociception mechanism and therapeutics. Studies in rodents show that reflexes (observed as withdrawal, avoidance, and changes in respiration/heart rate) occur in response to noxious stimulation (mechanical or chemical), which suggest activation of sensory neurons called nociceptors (Mense, 1977; Mense and Meyer, 1985). Therefore, clues about nociception can be studied in mammalian species with conserved mechanisms of nociception similar to those of humans, and those models provide useful
knowledge and insights in the control of pain states. However, in pre-clinical models of nociception our ability to derive the subjective component compromising pain has several restrictions. Differences between rodent and human response to nociceptive stimuli have been reported and can be attributed to differences in the anatomy of the nervous systems and input from emotional and cognitive centers of the brain (Mogil, 2009). fMRI conducted in rats that receive noxious stimulation of the colon show similar activity in brain regions corresponding to patients with irritable bowel syndrome (Johnson et al., 2010). Hence, despite the differences between two species, animal models are useful in developing the framework to improve strategies in clinical research.

Pain that is reoccurring and persistent over a prolonged period of time after the resolution of an eliciting event is considered an aberration of the sensory system that transmits pain and is recognized as a serious disease in clinical settings. Therefore, a classification of pain has been established that creates a distinction between the physiological function of pain (acute) and the clinical problem of pathological pain (chronic) (Woolf, 2010). The nociceptive system involves a rapid activation of high threshold afferent fibers (see section 1.2), which is commonly accompanied by a withdrawal reflex response in animals (Hargreaves et al., 1988). Under physiological conditions the activation of the high threshold afferents eventually subsides after the eliciting stimulus has dissipated (Kohllöffel et al., 1991). If tissue damage occurs, a response that involves tenderness, inflammation, and hypersensitivity to the injured site is evoked (Kilo et al., 1994). This condition can be considered acute or chronic, depending on the severity and exposure to tissue damage (Koltzenburg et al., 1994). However, in
some cases the damaged tissue is repaired but high threshold afferents fibers along with low threshold afferents remain activated and interpret non-noxious stimuli as nociceptive, a condition referred to as allodynia (Torebjörk et al. 1992). A similar condition occurs during hyperalgesia, defined as an increased nociceptive response to a stimulus which is normally painful (Meskey and Bogduk, 1994). Allodynia and hyperalgesia represent a sensory experience mediated by activation of nociceptors and is not protective. Instead, nociception is due to an altered nervous system and non-neuronal neighboring cells, such as glia, and it is referred to as pathological pain (Woolf, 2010). Pathological pain can occur after direct damage to the nervous system, as observed in patients with nerve injury or trauma that suffer from neuropathic pain. However, the abnormal sensitization of the nervous system can also arise in non-inflammatory or non-traumatic cases known as dysfunction pain. Dysfunctional pain includes visceral nociception associated with irritable bowel syndrome, in which an amplified sensory signal in the central nervous system and activation of low-threshold afferents contribute to the disease state (Ossipov et al., 2010). Patients that suffer from irritable bowel syndrome or interstitial cystitis lack abnormalities in the visceral structure or biochemical changes (inflammation) that could explain the recurrent pain. Therefore, the etiology and natural course of irritable bowel syndrome and interstitial cystitis remain unclear. However, in most cases, changes in the excitability of the sensory system (hypersensitivity) occur during the initiation of somatic (Simone et al., 1991; Treede et al., 1992) and visceral (Kolhekar and Gebhart, 1996) enhanced pain states. Hence, an understanding of the sensory system is required to decipher the complexity of persistent pain.
1.2 Somatic Sensory System

The peripheral (somatic) nervous system (PNS) encompasses the sensory regions of the skin and muscles that have their neuronal cell bodies in the dorsal root or the trigeminal ganglia (Basbaum et al., 2009). The somatic system interprets noxious stimuli by afferent nerve fibers comprising high-threshold unmyelinated C-fibers and thinly myelinated Aδ-fibers (D'Mello and Dickenson, 2008). Somatic pain transmission response to noxious stimuli is carried from primary afferents via nociceptors to second order neurons found mainly on lamina I and II of the spinal dorsal horn (D'Mello and Dickenson, 2008). Second order neurons then ascend through the lateral spinothalamic tract to the brainstem and continue to the supraspinal tract in the thalamus, hypothalamus, and amygdala (Figure 1.1 A) (Kuner, 2010). Also, tracts within the ventrolateral quadrant (spinothalamic, spinoreticular, spinomesencephalic, and spinohypothalamic) send nociceptive transmission to supraspinal sites (Kuner, 2010). Although the spinothalamic and the spinoparabrachial tracts are important for the ascending pain pathways, it is also important to realize that the maturation of the nociceptive pathways and different stages at which they synapse with targeted cells remains unknown (Hunt and Mantyh, 2001). Nevertheless, an important ascending pathway involved in pain originates from lamina I of the dorsal horn and project to the parabrachial area of the brainstem that then project to the amygdala and hypothalamus (regulates changes in heart rate and blood pressure).
A separate set of fibers called low threshold myelinated Aβ-fibers normally function as tactile sensors (D'Mello and Dickenson, 2008; Ma Q, 2010). In neuropathic pain, low threshold Aβ-fibers undergo a phenotypic switch and contribute to nociceptive transmission after peripheral-noxious stimuli (Neumann et al., 1996). In addition, Aβ-fibers have also been implicated in mediating mechanical allodynia by producing and releasing substance P (Neumann et al., 1996). The release of excitatory neurotransmitters such as glutamate and substance P cause the activation of postsynaptic glutamate receptors and the tachykinin receptor termed neurokinin-1 (NK1), respectively, that are involved in sensitization of second-order neurons; a process referred to as central sensitization.

Noxious stimuli can activate several cortical areas, such as the somatosensory cortex regions I and II, posterior parietal cortex, insula, prefrontal cortex, and anterior cingulate cortex (Apkarian et al., 2005). These areas are also involved in the descending control of pain. Moreover, a particular cortical area of interest is the anterior cingulate cortex (ACC) due to its link with the cognitive aspects of pain (Zhuo, 2007).

Descending inhibition and excitation are modulated by the spinal pathways of the dorsolateral funiculi and ventral/ventrolateral funiculi, respectively (Millan, 2002). Brainstem structures participate in the formation of descending pathways to the dorsal horn in a spinobulbar spinal loop that control pain transmission by numerous monoaminergic and peptide transmitters found in their spinal terminals (Tavares and Lima, 2002). The periaqueductal grey (PAG) forms part of the descending pain system and receives input from the amygdala, hippocampus, and hypothalamus (Bandler and
Shipley, 1994). Classical studies have shown that electrical stimulation of the PAG lead
to suppression of pain (Reynolds, 1969). Therefore, pain modulation has been linked with
descending pathways. In chronic pain, such as neuropathic pain, C-fibers exhibit
enhanced ectopic discharge and may be maintained by descending facilitation and
upregulation of spinal dynorphins (Porreca et al., 2002; Lai et al., 2006).

1.3 Visceral Sensory System

The internal organs of an animal body are collectively referred to as viscera.
Several differences in the pain transmitting pathway exist between the somatic and
visceral sensory system (discussed in section 1.4). A unique characteristic of visceral organs is their dual innervations that project to different regions of the central nervous system (CNS) (Figure 1.2 A-B) (Sengupta, 2009). For example, the distal colon is innervated by pelvic and/or splanchnic nerves (Figure 1.2B) (Jänig and Koltzenburg, 1990; Baron and Jänig, 1991; Sengupta and Gebhart, 1994). Thus, colonic afferents send signals via a dual nerve system from the periphery to the CNS. The gastrointestinal tract also contains a unique enteric nervous system that can auto regulate complex functions independent of the CNS.

Figure 1.2. Visceral sensory pathways. A) Spinal innervation of the visceral organs occurs via two nerves (Wall and Melzack’s Textbook of Pain 5th ed. 2006). B) Spinal innervation of the distal colon occurs via the splanchnic and pelvis nerves (Adapted from Brierly and Blackshaw, 2007).
Similarly, the urinary bladder has thoracolumbar innervations with sensory inputs extending up to the T10 level in humans (de Groat and Yoshimura, 2009). Moreover, the bladder has spinal innervations from the pelvic nerve (S2-S4) (Jänig and Koltzenburg, 1990; de Groat and Yoshimura, 2009). Therefore, the bladder and colon share sensory pathways and may be the cause of referred pain (discussed in section 1.5).

1.4 Somatic vs. Visceral Pain

Although there are correlations between somatic mechanoreceptor/nociceptors and visceral mechanoreceptors/nociceptors, it is clear that differences exist in processing of nociceptive information. To understand these differences this section will start by establishing basic properties of the somatic mechanosensitive afferent fibers, followed by the less characterized afferent fibers of the viscera. Somatic afferent fibers carry information to distinct lamina layers of the spinal cord (D'Mello and Dickenson, 2008). Most somatic afferent fiber terminals can be found in the marginal layer (lamina I) and substantia gelatinosa (lamina II). Somatic afferents consist of three classes of fibers distinguished by their conduction velocities and myelination; 1) Aβ-fibers are myelinated with a high conduction velocity that do not transmit nociceptive information under physiological conditions; 2) Aδ-fibers are lightly myelinated with a medium conduction velocity and a low mechanical activation threshold; 3) C-fibers have the lowest conduction velocity, are not myelinated, and transmit nociceptive information at high mechanical activation thresholds (D'Mello and Dickenson, 2008). Each of the classes has
subclasses that are based on mechanosensory, chemosensory, and nociception responses (Davis et al., 1993). Also, somatic afferents fibers can be classified by the location of their receptive fields and rates of adaption (i.e., slow adapting vs. fast adapting) (Reeh et al., 1987; Baumann et al., 1991). In addition, somatic afferent fibers can be distinguished by neurochemical markers and referred to as peptidergic or non-peptidergic (Silverman and Kruger, 1988; McCarthy and Lawson et al., 1989). Peptidergic pain transmitting neurons express substance P and calcitonin gene-related peptide (CGRP), whereas the nonpeptidergic pain transmitting neurons express glial cell derived growth factor (GDNF) (Lawson et al., 1996; Bradbury et al., 1998), ATP gated ion channel P2X3, and bind lectin IB4 (Hunt and Mantyh, 2001). Moreover, in mice, the nonpeptidergic population does not express TRPV1 (Zwick et al., 2002). Therefore, peptidergic fibers transmit pain information from somatic tissue after noxious stimulation by activators of TRPV1; acid, heat, and capsaicin (Holzer, 1988; Caterina et al., 1997). Although studies have shown that capsaicin sensitive (Yoshimura and de Groat, 1999; Dinis et al., 2004) and IB4 expressing (Zinck and Downie, 2008) fibers are responsible for changes in bladder function and pain, there is controversy surrounding the classification of visceral afferents and their encoding properties (Foreman, 2004).

Unlike the diversity of the afferent subclasses mediating somatic pain transmission, visceral afferents have conduction velocities that are limited to either small diameter unmyelinated C-fibers or thinly myelinated Aδ-fibers (de Groat WC and Yoshimura, 2009). However, similar to somatic afferents fibers, visceral afferent C-fibers and Aδ-fibers respond to chemical and/or thermal stimuli in addition to mechanical
stimulation mediating nociceptive transmission (Blackshaw et al., 2007). Also, similar to somatic afferent fibers, visceral afferent fibers are classified based on the layer of the viscera containing their receptive field, the type of mechanical stimuli that elicits a response, modalities (mechanoreceptors, chemoreceptors, nociceptors), neurochemical markers (peptidergic vs non-peptidergic) and rate of adaption to a continuous stimuli (Jänig and Koltzenburg, 1991; Sengupta and Gebhart, 1994; Bennett et al., 1996; Zagorodnyuk and Brookes, 2000; Sengupta, 2009). For example, Sengupta and colleagues (1994) observed that mechanosensitive bladder afferents that responded to non-inflammatory noxious stimulation consisted of both Aδ and C-fibers (Sengupta and Gebhart, 1994). However, most myelinated Aδ-fibers found in bladder afferents are mechanosensitive and half of unmyelinated C-fiber bladder afferents tended to be silent C-fibers that are activated mostly during chemical stimulation (Dmitrieva and McMahon., 1996). Immunohistochemical staining and patch clamp recordings demonstrate that the most common population of bladder afferent fibers have high threshold potentials and are assumed to be C-fiber afferents, whereas Aδ-fibers tend to be low-threshold mechanoreceptors (Vera and Nadelhaft, 1990; Yoshimura and de Groat, 1999). Indeed, several classical studies have revealed that C-fibers respond to cold stimuli or chemical stimuli, such as high potassium and capsaicin (Maggi et al., 1993; Wen and Morrison, 1995). Also, electrophysiologic measurements after urinary bladder distension (UBD) demonstrated that activation of bladder C-fibers occurred after UBD and/or chemical stimulation (Ustinova et al., 2007).
Although specialized endings found in somatic afferents have been extensively defined, the majority of information on specialized endings found in viscera is not known. However, the intestines have intramuscular arrays that are tension receptors (mechanoreceptors) that respond to passive stretch and contraction of the muscle (Phillips and Powley, 2000; Berthoud et al., 2004). In addition, the intestines have intraganglionic laminar endings and mucosal endings that are part of the enteric system (Sengupta, 2009). Whether these colonic mechanoreceptors transmit visceral nociceptive information is not fully understood due to difficulty of electrophysiological recordings from the various visceral tissue layers (Berthoud et al., 2004).

Visceral afferents terminate in areas of the spinal cord that overlap with the terminals of somatic afferents (Figure 1.4) (Sengupta, 2009). These areas include the superficial layers of the spinal dorsal horn lamina I-II (Christianson et al., 2009). Pain transmitting visceral afferents entering the dorsal horn represent less than 10% of primary afferents projecting to the spinal cord (Christianson et al., 2009). The poor representation of sensory input from the visceral organs is compensated by collateral branches/arborization that terminate in the superficial laminae, lamina V, and X (Figure 1.3) (Sugiura et al., 1989). Therefore, diffuse activation of second order neurons in the spinal cord lamina may occur as a consequence of arborization of visceral afferents, and may help explain the diffuse manifestation of visceral nociception and referred pain (see section 1.5) (Sugiura et al., 1993). Moreover, the extensive terminal arborization of the visceral afferents can extend rostrocaudally for several spinal segments (Nadelhaft and Booth, 1984).
Although visceral nociceptive pathways share several tracts of the somatic pathways, the bulk of nociceptive transmission may occur elsewhere (Christianson et al., 2009). Somatic pain transmission from the dorsal horn of the spinal cord travels to the brain via the ventrolateral quadrant white matter (Kuner, 2010). Also, tracts within the ventrolateral quadrant (spinothalamic, spinoreticular, spinomesencephalic, and spinohypothalamic) send nociceptive transmission to supraspinal sites (Kuner, 2010). In
visceral nociception the spinal dorsal column seems to be an important visceral nociceptive pathway from the spinal cord to the nucleus gracilis of the medulla (Figure 1.1B) (Sengupta, 2009). Hirshberg et al. (1996) demonstrated that a limited midline myelotomy relieved pain due to colon cancer for the remaining survival period of a patient (Hirshberg et al., 1996). Thus, the dorsal midline is one of at least two ascending pathways important to visceral nociceptive transmission (Christianson et al., 2009).

1.5 Cross-organ sensitization

Sensitization of urinary bladder afferent pathways to mechanical and chemical stimuli after irritation/inflammation of the colon, or vice-versa, are examples of cross-organ sensitization (Arvidsson et al., 2006). Recent studies have addressed the mechanisms mediating cross-organ sensitization termed clinically as referred pain. Patients diagnosed with irritable bowel syndrome (IBS) tend to also have symptoms of interstitial cystitis/painful bladder syndrome (PBS) and vice versa (Alagiri et al., 1997; Francis et al., 1997).

Although the exact mechanism that gives rise to cross-organ sensitization pathway is not clear, four possibilities have emerged that could help explain the generation of cross-organ sensitization. The first possibility is that cross-organ sensitization pathway may be linked with the convergence of afferent pathways from the bladder and bowel onto the same second order spinal neuron (Figure 1.4A) or via spinal interneurons (Figure 1.4B) (Willis et al., 1999). The second possible pathway involves
the divergence of primary afferents from the bladder and colon onto a single neuron in the dorsal root ganglia (Figure 1.5A) (Christianson et al., 2006). The third possible pathway can occur inside the nerve-bundles that carry the visceral afferents from distinct visceral organ, in which chemical (i.e., neurotransmitters) and electrical interaction can occur between injured and non-injured primary afferents (Figure 1.5B). The last proposed pathway of cross-organ sensitization is that sensitization of primary afferent fibers from an injured organ may transmit pain information via axon collaterals to the autonomic neurons in the prevertebral ganglia that may innervate the colon (Figure 1.5B)(Kaleczyc et al., 2003; Brumovsky et al., 2009). Since glutamate is the principle neurotransmitter of primary afferents from the colon and bladder (Broman et al., 1993), reduced extracellular glutamate via the over-expression of GLT-1 in the central nervous system may be useful to mitigate cross-organ sensitization.

Figure 1.4. Cross-organ sensitization. Cross-organ sensitization can occur A) by visceral afferent terminals transmitting signals to the same second order neuron or B) by transmitting signal to an interneuron that relays sensory information to two independent second order neurons (Adapted from Brumovsky and Gebhart, 2010).
Figure 1.5. Dichotomizing afferents and autonomic-induced cross-organ sensitization. A) Cross-organ sensitization by dichotomizing primary afferent onto the same sensory neuron or B) via autonomic and afferent interaction (Adapted from Brumovsky and Gebhart, 2010).

Animal models have been developed to demonstrate that colonic irritation/inflammation leads to sensitization of the urinary bladder afferents (Ustinova et al., 2007). Qin et al. showed that cross-organ sensitization of lumbosacral spinal neurons receiving input from the bladder occurs after colonic inflammation in rats, demonstrating an observed relationship between neuronal hyperexcitability and cross-organ sensitization (Qin and Foreman, 2004). Moreover, inflammation of the bladder by cyclophosphamide, via renal excretion of acrolein (Vizzard et al., 1996; Bjorling et al., 2007), resulted in enhanced visceromotor response to colo-rectal distension (Bielefeldt et al., 2006). Colonic inflammation can also alter the function of the bladder by enhancing micturition patterns (Lamb et al., 2006; Noronha et al., 2007) in mice.
As stated before, the bladder and colon have afferent fibers that project and terminate in the same regions of thoracolumbar and lumbosacral levels of the spinal cord. However, somatic afferents can also be found in the same lamina regions (Figure 1.6). Hence, the convergence of the afferents suggests that persistent sensitization of the second order neurons in the dorsal horn can be activated by either viscera or somatic tissue. For example, studies have demonstrated that somatovisceral convergence occurs in rats that have undergone nerve injury can develop visceral hypersensitivity of the colon (Randich et al., 2006). Although the exact mechanisms that mediate somatovisceral convergence is beyond the scope of this dissertation, the current theory suggests that overlap of the pain transmitting neurons from visceral and somatic afferents in the dorsal horn of the spinal horn are directly responsible for mediating hyperalgesia and that the mechanism may also involve supraspinal neurons (Kaddumi et al., 2007). Therefore, spinal mechanisms are also of interest in targeting the development of cross-organ sensitization. Furthermore, the spillover of glutamate onto neighboring synapses as observed by Nie et al. (2009) may help explain the phenomena of referred pain of visceral organs via an enhanced activation of pain mediators (Nie et al., 2009)(see section 1.2).
The focus of this dissertation is to establish the potential importance of augmenting the activation of glutamate transporters resulting in reduced extracellular glutamate, as a mechanism to mitigate visceral nociception. Marked implications for potential therapeutics and modulation of cross-organ sensitization via a mechanistic approach would be the result. This dissertation will investigate whether enhanced activity of the predominant glutamate transporter GLT-1 can effectively limit colon to bladder referred nociception.

1.6 Behavior Model

Nociceptive responses in the mouse and rat models are commonly measured by quantitating brain stem reflexes that include flexion/withdrawal from noxious stimuli.
(somatic), cardiovascular changes (i.e., changes in blood pressure, respiration, and heart rate) (somatic and visceral), and regional or generalized muscle contractions (visceral) (Hargreaves et al., 1988; Ness and Gebhart, 2001; Wilson et al., 2001; Arvidsson et al., 2006). Indeed, high frequency stimulation of C-fibers increases the flexion withdrawal reflex in rats (Woolf and Thompson, 1991). These reflexes collectively are termed “pseudffective responses” (Fioramonti et al., 2007).

Early behavioral models to assess visceral noiception in conscious rodents required the injection of acid or formalin into the abdomen and measurement of writhing response. The disadvantage of the writhing model was that the measured nociception may not be viscera-specific (Flower et al., 1985; Ness and Gebhart, 1990). Therefore, other models were developed that focused on visceral nociception. Currently, the most commonly used technique to measure evoked visceral nociception is by recording abdominal contractions during colo-rectal distention (CRD) in awake rodents (Larsson et al., 2003; Kamp et al., 2003), which are produced by lumbosacral-bulbo-spinal loops (Ness and Gebhart, 1988). Similar approaches have been used to elicit visceromotor responses (VMR) in response to distension of the bladder (Ness et al., 2001; Ness and Elhafni, 2004), esophagous, and stomach (Bielefeldt and Davis, 2008). Ness and colleagues pioneered the model for urinary bladder distension (UBD) in rats and mice and found a correlation between graded bladder distension pressures and evoked visceromotor responses that are inhibited by analgesics (Ness et al., 2001; Ness and Elhafni, 2004; Blatt et al., 2009). Based on these reliable rodent models of visceral nociception, a repeatable and quantitative measurement can be obtained to determine the
effect of treatments on bladder nociception. Also, studies in rodents revealed that CRD (at 60 mm Hg) elicits an increase (insula, somatosensory cortex, and anterior cingulate cortex) or decrease (thalamus, hypothalamus, pons) in cerebral blood flow in regions seen in human visceral pain studies (Wang et al., 2008). Moreover, a recent study using fMRI, indicated that rats that undergo CRD show similar activity in brain regions corresponding to IBS patients (Johnson et al., 2010).

1.7 Chronic Visceral Pain

Chronic visceral pain is a frequent cause of morbidity in the general population, affecting up to 15-20% of the US population and resulting in healthcare costs of 41 billion each year in eight leading healthcare economies (NIH, 2002). Opiates are not effective in treating chronic visceral pain long-term (Ballantyne and La Forge, 2007). Unfortunately, clinicians overuse of opioid therapy for chronic visceral pain management often leads to addiction, drug tolerance, prescription analgesic abuse, and hyperalgesia (Ballantyne and Mao, 2003). Moreover, mu(µ)-opioid agonists induce euphoria, respiratory depression or gastrointestinal transit inhibition and some kappa-opioid receptor agonists lead to dysphoria and sedation (Rivière, 2004). Therefore, opioid based treatment for visceral pain is suboptimal. Effective management of chronic visceral pain remains a difficult task to accomplish, in part due to the lack of basic understanding of the novel aspects of the visceral pain pathway.
A sensitization of nociceptive pathways from the bladder and colon is often elicited in animal models via tissue inflammation that ultimately results in hyperalgesia or allodynia. Consequently, the changes that occur during central sensitization are caused by an increase in the activity of primary afferents that trigger the enhanced release of neurotransmitters (e.g., glutamate) resulting in activation of receptors and ion channels located on second order neurons (Wolf and Thompson, 1991; South et al., 2003). Classical studies showed that increased dorsal horn neuron activity by repeated C-fiber stimulation was mimicked by L-glutamate or glutamate receptor activation (Gerber et al., 1989) and blocked by NMDA antagonists (Thompson et al., 1990). Studies in rodents have demonstrated that administration of a noxious chemical or distension of the colon in neonatal period leads to enhanced visceral sensitivity in adult animals (Tachibana et al., 2001; Lin and Al-Chaer, 2003; Blom et al., 2006). Also, early maternal separation of rodent pups is an accepted animal model for irritable bowel syndrome (O’Mahony et al, 2009; Gosselin et al., 2010). Similar studies in the bladder have demonstrated that changes occur in the adult bladder due to neonatal bladder inflammation (Randich et al., 2006; DeBerry et al., 2010). Therefore, it is postulated that neuronal plasticity related to neonatal insult occur as a consequence of interference with the physiological development of visceral afferents (Veenema and Neumann., 2008).

An animal model for chronic visceral nociception produced in adult rodents usually involves inflammation of the visceral organ. Dextran sodium sulphate (DSS) causes prolonged colon inflammation and has been employed as a rodent model of chronic colitis (Okayasu et al., 1990). For example, three cycles of 5 days of 5% dextran

20
sodium sulphate (DSS) followed with 2 weeks of regular water induced visceral hyperalgesia in mice (Verma-Gandhu et al., 2007). Although the mechanism by which DSS causes colitis is unknown, there is evidence that resident bacteria may be involved, and there is increased activity of the immune system (Wirtz et al., 2007; Nagalingam et al., 2010).

The haptenizing compound 2,4,6 trinitrobenzene sulfonic acid (TNBS) is also used as a model for irritable bowel disease. A single intracolonic injection of TNBS into the colon of rodents produces a prolonged decreased pain threshold to colorectal distension (Diop et al., 2002). CD4\(^+\) T-cells are important for establishing chronic TNBS colitis (Wirtz et al., 2007) and results in an inflammatory-induced thickening of the colon wall (Neurath et al., 1996).

Systemic administration of cyclophosphamide serves as a model of interstitial cystitis during bladder inflammation conditions. This substance is metabolized into acrolein by the liver and when it collects in the bladder leads to prolonged bladder inflammation (Vizzard et al., 1996; McDonald et al., 2003). Lai et al., showed that chronic inflamed animals after cyclophosphamide treatment increased ERK1/2 phosphorylation (p-ERK1/2) within dorsal horn neurons (L6), which may be involved in sensitization of postsynaptic neurons (Lai et al., 2011). Changes in the activity of postsynaptic neurons (i.e., receptors, transcription factors, trafficking) and enhanced synaptic efficacy are described as central sensitization, and are the basis for several models of chronic pain. Both rodent and human studies have established the importance for spinal glutamate receptors in central sensitization after visceral insult (Cervero et al.,
1995; Peles et al., 2004; Zhou et al., 2009; Willert et al., 2004). Therefore, glutamate and activation of its receptors are implicated in the enhanced activation of second order neurons that represents central sensitization, and strategies to reduce extracellular glutamate may provide important anti-nociceptive effects.

1.8 Glutamate Receptors

Both somatic and visceral sensory systems use glutamate as the main excitatory neurotransmitter for physiological and pathological sensory transmission. Hence, studies have explored effective treatments of somatic and visceral nociception by targeting the glutamate receptors (Olivar et al., 1999, Traub et al., 2002; Galan et al. 2004) and the subsequent intracellular activation of kinases (Galan et al., 2003). However, inhibition of glutamate receptors has adverse consequences. For example, glutamate receptor inhibition is linked with hypermetabolism of cortical neurons (Hargreaves et al., 1994) and intrathecal treatments show motor side effects (Lee et al., 2006). Therefore, visceral pain research has focused on the efficient removal of the excess glutamate that activates glutamate receptors and contributes to visceral nociception associated with colo-rectal distension in mice (Lin et al., 2009). Overall, development of effective treatment of somatic and visceral pain is hindered by the lack of understanding of the pathophysiological mechanism(s) involved in chronic states of pain. Glutamate is the main candidate for exploring these obscure mechanisms and generating effective treatments.
Glutamate receptors are key mediators of transmission of nociception input and are classified as ionotrophic or metabotropic. The ionotrophic glutamate receptors are further divided into three types called N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and 2-carboxy-3-carboxymethyl-4-isopropylpyrrolidine (kainate) receptors (Tao, 2010). The ionotrophic glutamate receptors share several structural features that place them in the same family due to their shared transmembrane topology. All three ionotrophic glutamate receptors have been linked to visceral pain (Zhou et al., 2009; Liu and Salter, 2010). Thus, their function is critical for understanding the effects of reduced extracellular glutamate via over-expressing GLT-1 on the nociceptive response.

NMDA receptors are calcium permeable and are blocked by magnesium ion under physiological conditions. The NMDA receptor consists of the subunits NR1, NR2A-D, and NR3A-B (Figure 1.7) that determine the pharmacological and physiological properties of the receptor (Zou et al., 2000; Guo et al., 2002). The distribution of the subunits can vary in the spinal cord in rodents (Gogas, 2006). The NR1 is distributed throughout the laminae of the spinal cord in rats (Iwata et al., 2007). Also, NR2A are found in most regions of the spinal cord except lamina II (Nagy et al., 2004). However, the exact distribution of the NR2 remains widely disputed (Boyce et al., 1999). Nevertheless, lamina II is occupied by NR2B and this subunit is restricted to the superficial dorsal horn (Nagy et al., 2004; Boyce et al., 1999). Studies have revealed that the NR2B may have an important role in neuropathic (Matsumura et al., 2010; Katano et al., 2011) and visceral pain (Chang et al., 2010; Peng et al., 2010) by contributing to
long-term potentiation of primary afferents. Moreover, studies by Zhou et al. (2009) showed that TNBS induced colitis increase expression of the NR1 in the spinal cord of rats and may contribute to central sensitization (Zhou et al., 2009). Also, Nie et al. demonstrated that impaired glutamate uptake by GLT-1 increased the activity of NMDA receptors and could contribute to nociceptive transmission (Nie and Weng, 2009).

![NMDA Receptor Diagram](image)

**Figure 1.7. NMDA receptor.** NMDA subunits found in (A) peripheral tissue or in the (B) dorsal horn (Adapted from Petrenko et al., 2003).

The AMPA receptor consists of four subunits that are calcium permeable (GluR1-4). The most abundant of the subunits are GluR1 and GluR2. The subunits are assembled in the dendrites of neuronal cells (Wang et al., 2010). The AMPA subunit mRNA are localized into dendrites and translation occurs after neuronal activity (Man et al., 2000). Once the AMPA subunits are formed they diffuse into the cytosol and can be maintained
or recycled back to the plasma membrane (Tao, 2010; Santos et al., 2009). Since AMPA receptors are highly concentrated in the spinal dorsal horn and activation results in calcium influx that leads to a strengthening of synaptic transmission, it is hypothesized that AMPA receptors may contribute to visceral pain states (Galan et al., 2004). A study by Galan et al. showed that an increased amount of GluR1 in the membrane and decreased amount in the cytosol occurred after intracolonic capsaicin treatment, and this correlates to visceral hyperalgesia (Galan et al., 2004). Moreover, studies have shown that the GluR1 subunit contributes to synaptic activity in the lumbosacral dorsal horn by facilitating calcium influx to elicit potentiation after a noxious stimulation and may mediate cross-organ sensitization (see section 1.10)(Peng et al., 2011).

Figure 1.8. Comparison of NMDA and AMPA activation during normal conditions and persistent inflammation (Adapted from Tao, 2010).

Also, studies of somatic pain revealed that formalin injections into the hindpaw of rats increased GluR1 levels in the plasma membrane (Zhou et al., 2001). However, other
studies suggest that changes in AMPA receptor subunit expression and trafficking are based on the type of noxious stimuli. For example, persistent whole-body inflammation by complete Freund’s adjuvant (CFA) systemic injection produces a significant decrease of calcium impermeable GluR2 in the neuronal membrane and increase in the cytosol (Figure 1.8). At the same time calcium permeable GluR1 subunits may be shuttled to the membrane. As a result, subunit trafficking of the AMPA receptors in the spinal neurons may indicate that they are responsible for the maintenance of central sensitization during nociception due to inflammation by establishing an increased calcium influx that enhances second order neuronal activity (Tao, 2010).

The molecular mechanisms that alter the expression of AMPA receptors after a noxious stimulus are the subject of recent studies. Patch-clamp recordings of rat dorsal horn neurons demonstrated that during the CFA maintenance phase the number of calcium permeable AMPA receptors found in second order neurons increased during inflammatory pain (Park et al., 2009). Also, evidence suggests that phosphorylation of serine residues found in the c-termini of the AMPA subunits may play a role in increasing neuronal activity by contributing to calcium influx (Figure 1.8) (Leever et al., 2003).

NMDA and AMPA can interact to modulate calcium influx and regulate trafficking. NMDA receptors may alter the expression of the AMPA receptors due to the calcium influx after NMDA receptor activation that activates kinases that phosphorylate the AMPA subunits (Figure 1.8)(Tao, 2010). Also, under physiological conditions NMDA receptors can remain open after binding glutamate and allow calcium to enter the
cell, however, the AMPA receptors can modulate calcium influx after binding glutamate (Tao, 2010). Hence, the increase in calcium influx leads to activation of several kinases, but in particular the ERK1/2 pathway involved in mechanisms of hypersensitivity (Corrow et al., 2009), inflammatory pain (Qiao et al., 2008), and central sensitization (Galan et al., 2003).

These studies taken together suggest that glutamate receptors are relevant to both visceral and somatic pain. Although studies show that glutamate receptor antagonists attenuate chronic and acute somatic and visceral nociception (Rustioni, 2005; Ultenius et al., 2006; Larsson, 2009), clinical utility of glutamate antagonist are limited because of severe adverse side effects (Vranken et al., 2005; Cvrcek et al., 2008). Therefore, an alternative approach to limit glutamate receptor activation is to enhanced glutamate uptake. This approach decreases NMDA/AMPA receptor activation and may ultimately mediates anti-nociception (Li et al., 1999; Lin et al., 2009). The novel approach of decreasing glutamatergic neurotrasmission by increasing the activity of GLT-1 glutamate transporters is the focus of this dissertation.

1.9 Glutamate Transporters

Five sodium dependent glutamate transporters have been discovered and have similar crystal structure that contains 8 transmembrane domains and exist as trimers (Schousboe and Waagepetersen., 2006). Two glutamate transporters are located in astrocytic cells and are called GLAST and GLT-1 (the human homologues EAAT1 and
EAAT2, respectively; see table 1.1). The distribution of GLAST and GLT-1 in astrocytes is not uniform and the transporters are mostly expressed near the synapse (Figure 1.9) (Chaudhry et al., 1995; Trotti, 2002). Therefore, both transporters participate in reducing the amount of glutamate that is available for glutamate receptors (Weng et al., 2007). Two of these transporters, EAAC1 (or EAAT3) and EAAT4, are found primarily in neurons (Schousboe and Waagepetersen, 2006). Interestingly, in most neurons EAAC1 and EAAT4 are distributed in the post-synapse spine and perisynaptic region (Beart and O'Shea, 2007). EAAT5 is a member of the glutamate transporters found only in rod photoreceptors and bipolar cells in the retina (Boehmer et al., 2005).

<table>
<thead>
<tr>
<th>Human</th>
<th>Rodent</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAAT1</td>
<td>GLAST</td>
</tr>
<tr>
<td>EAAT2</td>
<td>GLT-1</td>
</tr>
<tr>
<td>EAAT3</td>
<td>EAAC1</td>
</tr>
<tr>
<td>EAAT4</td>
<td>EAAT4</td>
</tr>
<tr>
<td>EAAT5</td>
<td>EAAT5</td>
</tr>
</tbody>
</table>

Table 1.1 Human glutamate transporter nomenclature and rodent homologues.

GLT-1, however, is the physiologically predominant transporter responsible for removal (uptake) of excess glutamate in the extracellular space (Danbolt, 2001; Schousboe and Waagepetersen, 2006). Knockdown of GLT-1 leads to increased levels of extracellular glutamate, neurodegeneration, and paralysis in rats (Rothstein et al., 1996).
Also, downregulation of GLT-1 in rats resulted in reduced neuronal survival after ischemia (Rao et al., 2001). Inhibition of the GLT-1 transporter, via i.t. TBOA or DHK, in rats results in elevated nociceptive behaviors (Liaw et al., 2005).

Induction of GLT-1 expression *in vitro and in vivo* seems to be modulated by protein kinases (PKC), growth factors (NF-κB), and glutamate (González and Robinson, 2004). Under physiological conditions, glutamate transporters function as symporters co-transporting 3 molecules of Na\(^+\) and a proton with glutamate. Then a K\(^+\) ion is counter-transported causing the transporter to re-expose the glutamate binding sites to the extracellular space (Figure 1.10) (Anderson and Swanson, 2000). Therefore a cycle is established by astrocytes to maintain extracellular glutamate concentrations at a range of 1-4 µM in the brain (Lerma et al., 1986; Nyitrai et al., 2006). However, the exact concentration of glutamate found in the extracellular space is not known and lower concentrations of glutamate are often reported (Herman and Jahr, 2007).

![Glutamate transporters (EAAT1-3) found in astrocytes and neurons](image)

**Figure 1.9.** Glutamate transporters (EAAT1-3) found in astrocytes and neurons (Adapted from Dunlop, 2006).
Astrocytes historically were defined as cells that clean up the extracellular space and provide substrates necessary for neuronal function. Therefore, the purpose of
glutamate inside an astrocytic cell was hypothesized to enter the glutamate-glutamine cycle. Glutamate is metabolized in astrocytes into glutamine by glutamine synthetase (Figure 1.11) (Anderson and Swanson, 2000). The glutamine is then released and transported into neurons and converted back to glutamate.

Neurons then metabolized the glutamine back to glutamate (Anderson and Swanson, 2000). Thus, astrocytes are active participants in synaptic activity and maintain a viable microenvironment for neurons. Studies have indicated that GLT-1 is the predominant glutamate transporter responsible for 90% of glutamate uptake during neurotransmission (Kanai and Hediger, 2003). Furthermore, Marcaggi et al. demonstrated that glutamate spillover from the synaptic cleft of cerebellar granule cells was prevented by GLT-1 glutamate transporters (Marcaggi and Atwell, 2006). Hence, glutamate transporters are responsible for reducing exitotoxicity. Studies have revealed the importance of efficient glutamate uptake by glutamate transporters in several disease states, including ALS, Huntington’s Disease, and chronic pain. Increased expression of the glutamate transporters GLAST and GLT-1 correlates with increased expression of glutamine synthetase (Figiel and Engele, 2000; Rauen and Wiessner, 2000). Moreover, increased extracellular glutamate concentration due to spinal cord injury or ischemia, results in increased expression of glutamine synthetase (Petito et al., 1992; Benton et al., 2000). Although extremely high concentrations of extracellular glutamate reduce the expression of glutamate transporters in astrocyte cell cultures (Lehmann et al., 2009), the concentration of glutamate (>1 mM) used in cell culture studies may not reflect the smaller changes in extracellular glutamate concentration that occur in chronic pain.
conditions (Harris et al., 2009). Since glutamate is the predominant excitatory neutransmitter of postsynaptic neurons, interest has developed in regulating glutamate transporters, such as GLT-1, to mitigate chronic pain.

1.10 GLT-1 role in pain

It is known that dysregulation of glutamatergic systems (i.e., receptors and transporters) underlies many aspects of the etiology of chronic pain in animal models (Larsson and Broman, 2010; Lin et al., 2009). Peripheral nerve injury is associated with increased release of glutamate from primary afferents (Sluka and Willis, 1998; Kawamata and Omote, 1999). Given that glutamate transporters are responsible for efficient removal of glutamate from the extracellular environment, these molecules represent potential key modulators of pain transmission. Indeed, attenuation of the activity of astrocytic glutamate transporters are associated with several models of somatic pain (Tao, 2005; Sung et al., 2003; Tawfik et al., 2008). The importance of glutamate transporters in regulating glutamatergic systems in the setting of visceral pain has not been well studied.

Nevertheless, recent studies have shown that substances that augment glutamate transporter function reduce somatic (Hu et al., 2010) or visceral pain (Lin et al., 2009; Yang and Roman, 2011), and inhibitors or blockers of the glutamate transporters increase nociception (Lin et al., 2009; Gosselin et al., 2010; Ramos et al., 2010; Hu et al., 2010). Yaster et al. showed that blocking GLT-1 via selective inhibitors, injected intrathecally, led to a dose-dependent nociceptive behavior in rats (Yaster et al., 2011).
Moreover, the same study concluded that applying GLT-1 inhibitors topically on the spinal cord increases extracellular glutamate concentration likely responsible for persistent activation of glutamate receptors that mediate sensory hypersensitivity. Therefore, GLT-1 may play an important function in modulating the process in which central sensitization occurs in spinal neurons via glutamate.

Pharmacological agents that increase glutamate uptake, such as riluzole (Azbill et al., 2000), can reduce nociception following spinal cord injury (Hama and Sagen, 2011). Gosselin et al (2010) showed that visceral hypersensitivity in adult rats (8-10 weeks old), due to maternal separation, is reversed after systemic injection of riluzole (Gosselin et al., 2010). Moreover, the same study showed that intrathecal injection of DL-threo-β-benzyloxyaspartate (TBOA), a glutamate transporter inhibitor, reverses the anti-nociceptive effect of riluzole (Gosseline et al., 2010).

Similarly, ceftriaxone (CTX) is a β-lactam antibiotic that when administered to rodents produces GLT-1 over-expression (Rothstein et al., 2005). It is linked with neuroprotective effects in vitro and in vivo in diseases such as amyotrophic lateral sclerosis (ALS) and epilepsy (Rothstein et al., 2005; Sheldon and Robinson, 2007). Studies suggest that the mechanism that ceftriaxone utilizes to over-express GLT-1 is via nuclear translocation of p65 and activation of NF-κB that binds to the GLT-1 gene promoter region (González and Robinson, 2004). It has been suggested that CTX may be used to increase GLT-1 expression in astrocytes and improve the removal of extracellular glutamate.
The time course for GLT-1 over-expression is optimal after a 1 week treatment with daily CTX administration in mice (Chu et al., 2007). Moreover, the same study showed that GLT-1 over-expression peaks after 3 days and maintains a stable elevated expression at 7 days. The study also observed that the GLT-1 mRNA increased during the seven day period.

Alternative methods of over-expressing GLT-1 have emerged. Gene therapy is an emerging area for treating several neurobiological disorders (Beutler, 2010; Mason et al., 2011). A study by Maeda et al. showed that gene delivery of GLT-1 by an adenovirus was successful in mitigating nociception in animal models of neuropathic pain in rats (Maeda et al., 2008). Adenoviruses, however, are highly immunogenic, target multiple cell types, and often lead to secondary effects (Lai et al., 2002). Therefore, a translationally superior approach has emerged by using adeno-associated viruses (AAV) (Berns and Linden, 1995). An AAV genome infection can persist as episomes for the life of a non-dividing cell and give high levels of transgene expression for years. As a result, AAV transduction can give a sustained expression of the desired gene. Moreover, recent studies have demonstrated that the adeno-associated virus serotype 9 can successfully transduce astrocytic cells (Liu et al., 2005; Foust et al., 2009; Lawlor et al., 2009). Hence, virally mediated GLT-1 gene delivery to a specific site has the potential to localize GLT-1 over-expression and its use will be explored in this dissertation.
1.11 Problem Statement

The primary objective of the present work is to establish whether nociception emanating from a visceral organ (bladder) is attenuated by the strategy of glutamate transporter GLT-1 over-expression. The effect of GLT-1 over-expression on nociception associated with urinary bladder distension had not been studied. Thus, an approach to decrease glutamatergic tone by increasing expression of GLT-1 may be beneficial in the relief of chronic visceral pain disorders.

The second objective of this dissertation will focus on three visceral nociception models: interstitial cystitis, irritable bowel syndrome, and cross-organ sensitization (colon to bladder). Patients that receive cyclophosphamide as treatment for cancer develop interstitial cystitis; hence, animal models of interstitial cystitis have been developed that induce visceral hypersensitivity via repeated exposure of the bladder to acrolein (Vizzard et al., 1996; Bjorling et al., 2007; Lai et al., 2011). Also, patients that suffer from irritable bowel syndrome report childhood trauma; therefore, early life stress due to exposure of an irritant to the colon is a useful animal model of irritable bowel syndrome (O’Mahony et al., 2008; Cameron et al., 2008; Christianson et al., 2008). Moreover, the colon has primary afferents that terminate in spinal segments that overlap to those of the bladder (Sugiura et al., 1989; Brumovsky and Gebhart, 2010). In human populations that suffer from interstitial cystitis, 34.5% develop irritable bowel syndrome (Nickel et al., 2010). A similar condition is observed in rodent models, it has been documented that induced cystitis can result in colon hypersensitivity (Brumovsky et al.,
2009) and that colon inflammation elicits bladder hyperactivity (Pezzone et al., 2005; Lamb et al., 2006). This dissertation determines whether the beneficial effect of GLT-1 over-expression can be extended to these visceral nociception animal models.

The third objective of this dissertation will examine the site of action in the CNS that mitigates the visceromotor response to urinary bladder distension via over-expression of GLT-1. Although studies show that supraspinal regions and the spinal cord are involved in visceral nociceptive processing (Sikandar et al., 2011), the effect of GLT-1 over-expression in both these sites after CTX treatment is not clear. In models of colorectal distension, intracisternal administration of DHK did not reverse the effects GLT-1 over-expression (Lin et al., 2011). Therefore, this study will determine if a similar effect is observed in our model of urinary bladder distension.

The fourth objective of this dissertation aims to establish whether the over-expression of GLT-1, via ceftriaxone, alters the trafficking of the GluR1 subunit to the membrane of post-synaptic terminals after persistent inflammation of the bladder. Several reports implicate trafficking of AMPA subunits and changes in the AMPA channel permeability to Ca$^{2+}$ with activation of intracellular mechanisms that may be linked to somatic and visceral nociception states (Galan et al., 2003 and 2004; Katano et al., 2008; Kopach et al., 2011). Therefore, there is an increased interest in the role of AMPA during visceral nociception and the early removal of glutamate from the synaptic cleft by GLT-1 over-expression.

The final objective of this dissertation is to use the novel approach of gene therapy to attenuate visceral nociception by an adeno-associated virus serotype 9 (AAV9)
carrying the glutamate transporter protein gene. Previous studies show that adenoviruses that carry the GLT-1 protein gene can transduce cells and attenuate nociception in animal models of neuropathic pain (Maeda et al., 2008). Overall, this dissertation proposes that GLT-1 over-expression may result in increased glutamate reuptake, less trafficking of glutamate receptor subunits (GluR1) to the postsynaptic membrane, and attenuated visceral pain by ceftriaxone or gene therapy approaches.

1.12 Objectives and Research Questions

This dissertation seeks 1) to assess whether pain emanating from a visceral organ (bladder) is attenuated by GLT-1 over-expression; 2) whether visceral hypersensitivity due to persistent visceral inflammation is attenuated by GLT-1 up-regulation; 3) to determine if referral of visceral pain to another visceral organ can be mitigated by this approach; 4) to determine whether modulation of glutamate receptors might mediate the observed anti-nociception; and 5) whether visceral pain is reduced by adeno-associated virus mediated gene delivery of EAAT2; rodent homologue GLT-1.

Initial Hypothesis:

Hypothesis 1: Augmented glutamate transporter (GLT-1) expression mitigates urinary bladder distension (UBD) induced visceral nociception in acute and chronic models of visceral pain.

Hypothesis 2: GLT-1 over-expression mitigates bladder sensitization and cross-organ sensitization (colon to bladder).
Hypothesis 3: GLT-1 over-expression reduces visceral nociception by reducing spinal AMPA subunit (GluR1) trafficking.

Hypothesis 4: Adeno-associated virus serotype 9 mediated transduction of EAAT2 attenuates visceral nociception.

1.13 Significance of the Study

Study of the mechanism by which enhanced glutamate transporter activity mitigates visceral pain will likely have useful implications in developing drugs for treating hyperalgesic diseases, such as interstitial cystitis/painful bladder syndrome. Thus, pharmacological or viral gene delivery approaches may be viable treatment options for visceral pain and will improve our understanding of visceral nociception. This dissertation will expand on the current knowledge of GLT-1 over-expression as a mechanism to mitigate visceral pain and potentially lead to marked implications for potential therapeutics.

1.14 Definition of Terms

**AAV:** Adeno-associated virus that includes several serotypes (1-12) that can target and transduce cells (Beutler, 2010).

**Acute pain:** pain that is protective and involves activation of Aδ –fiber and C-fiber nociceptors; pain subsides without intervention (Boyce-Rustay et al., 2010).
**Allodynia:** A pain-free stimulus becomes a painful response (Basbaum and Bushnell, 2009).

**Central sensitization:** An increased in the perception of pain due to changes in input from sensory neurons and spinal dorsal horn neurons (Dubin and Patapoutian, 2010).

**Chronic pain:** an aberration of the pain sensory system and involves prolonged activation of C-fiber and A-fiber nociceptors. Chronic pain can be classified as a disease in the absence of ongoing injury; that lasts for several days (e.g., rodent chronic pain models) to years (e.g., humans) (Boyce-Rustay et al., 2010).

**Cross-organ sensitization:** The process of perceived pain sensation from a particular visceral organ due to proximity of nerve innervation in the same spinal segment as the actual affected visceral or somatic organ (Bielefeldt et al., 2006).

**Electromyography (EMG):** A recording of muscle contraction after a noxious stimuli by implanted electrodes (Basbaum and Bushnell, 2009).

**Hyperalgesia:** An enhancement of the nociceptive signaling pathways to environmental stimuli. (Dubin and Patapoutian, 2010).

**Nociception:** the neural process of encoding and transmitting noxious stimuli (Basbaum and Bushnell, 2009).

**Noxious Stimuli:** Damaging stimuli that include mechanical stimulation, chemical, and temperature changes that provoke a response (Basbaum and Bushnell, 2009).

**Receptor subunits:** Mostly found in ion channels and surround a central pore. These subunits can increase calcium permeability or change the duration a channel remains open (Basbaum and Bushnell, 2009).
Visceromotor response (VMR): A behavioral reflex-response that is elicited after a noxious stimuli to a visceral organ (Basbaum and Bushnell, 2009).
Chapter 2

GLT-1 over-expression attenuates bladder nociception and local/cross-organ sensitization of bladder nociception

2.1 Introduction

The National Institutes of Health estimates that up to 1 million people in the United States suffer from visceral pain associated with painful bladder syndrome or interstitial cystitis (Yoshimura and Birder, 2007). These conditions are accompanied by a myriad of symptoms (urinary frequency and urgency), and most notably prolonged pain. An emerging hypothesis suggests that bladder irritation and inflammation lead to release of chemical messengers that sensitize centrally projecting primary afferents that utilize predominantly glutamate as the neurotransmitter (Traub et al., 2007).

Glutamate, under physiological conditions, is rapidly cleared from the synaptic cleft by high-affinity, sodium-dependent glutamate transporters located in both neurons and glia (Hediger et al., 1999; Beart and O'Shea, 2007). The glial glutamate transporter GLT-1 is the quantitatively dominant glutamate transporter responsible for 90% of glutamate reuptake in the mammalian central nervous system and plays a major role in terminating synaptic transmission and protecting neurons from glutamate neurotoxicity.
Recently, the innovative approach of reducing extracellular glutamate by over-expressing the predominant glutamate transporter GLT-1 was found effective in animal models of both visceral (Lin et al., 2009; Lin et al., 2011; Yang and Roman, 2011) and neuropathic (Hu et al., 2009; Maeda et al., 2008) pain. Specifically, ceftriaxone (CTX), a β-lactam antibiotic, when administered to rodents produces GLT-1 over-expression and enhances glutamate uptake activity (Lin et al., 2011). These effects correlate with neuroprotective effects in vitro and in vivo in diseases such as amyotrophic lateral sclerosis and epilepsy (Rothstein et al., 2005; Sheldon and Robinson, 2007). The putative mechanism utilized by ceftriaxone to over-express GLT-1 is nuclear translocation of p65 and activation of NF-κB that binds to the GLT-1 gene promoter region (Lee et al., 2008). Thus, it is hypothesized that this novel approach to decrease glutamate extracellular levels by up-regulating glutamate transporter function may be beneficial in the relief of acute and chronic bladder pain.

Clinically, urinary bladder diseases are associated with viscero-visceral and viscero-somatic referral of pain; termed cross-organ sensitization (Brumovsky and Gebhart, 2009). For example, cross-organ sensitization occurs when lumbosacral spinal neurons receiving input from the bladder also receive sensory information after colonic inflammation (Qin et al., 2005). Evidence suggests that inflammation of the colon sensitizes the subsequent response of the bladder to noxious stimulation (Birder et al., 2007; Lamb et al., 2006; Noronha et al., 2007; Winnard et al., 2007), similarly, colon irritation increased the resting firing rate and the firing rate in response to distension of bladder afferents (Ustinova et al., 2006). As a consequence, colonic irritation or
inflammation enhances bladder nociception and altered urodynamic function (Peng et al., 2009), these responses were mediated significantly at the first central synapse of pain-transmitting neurons by the principle excitatory neurotransmitter glutamate (de Groat and Yoshimura, 2009). Thus, strategies to attenuate glutamatergic primary afferent neurotransmission may decrease referred pain.

This study will explore the effect of augmenting GLT-1 expression by 1 week ceftriaxone treatment on the visceromotor response to bladder distension. In addition, the effect of GLT-1 over-expression on 1) acute bladder irritation, 2) chronic bladder inflammation, and 3) cross-organ sensitization (colon to bladder) of the visceromotor response to bladder distension will be examined. The results reveal that pharmacological enhancement of GLT-1 expression and activity blunts the murine visceromotor response to bladder distension, acute bladder irritation, bladder inflammation, and cross-organ (colon to bladder) sensitization of the visceromotor response to bladder distension.

2.2 Methods

2.2.1 Animals. Two month old female FVB/N mice (20-25 g) were used for the experiments. Animals were housed singly on wood shavings with ad libitum access to food and water. The 12 hour light-dark cycle was maintained. Prior to experiments animals were fasted for 12 hrs to limit the fecal content of the colon; ad libitum access to water was maintained. Experiments were approved by Institutional Animal Care and Use
Committee from The Ohio State University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.2.2 Drug Administration. Ceftriaxone (CTX), a beta-lactam antibiotic, was prepared in saline and administered (200 mg/kg) intraperitoneally (i.p.) at 10:00 am for seven consecutive days; the control group received 0.9% saline (i.p.). This dosing regime of ceftriaxone selectively enhances GLT-1 expression in rodents (Rothstein et al., 2005). In some experiments, the selective GLT-1 antagonist dihydrokainate was administered at an effective dose (10 mg/kg, i.p.) (Liaw et al., 2005) 1 hour prior to urinary bladder distension; the control group received 0.9% saline (i.p.). In the study of colon-to-bladder sensitization of bladder nociception, intracolonic trinitrobenzene sulfonic acid (TNBS), a hapten, was administered (35 mg/mL; 50 µL) 1 hour before bladder distension in 1 week vehicle and 1 week CTX treated cohorts lightly anesthetized with isoflurane; the control group received the same volume of intracolonic vehicle (50% ethanol; diluent for TNBS). Intracolonic TNBS treatment was shown effective to sensitize urinary bladder afferents to mechanical and chemical stimuli (Ustinova et al., 2006). Another set of experiments examined 1) the effect of GLT-1 over-expression on augmented visceromotor response to bladder distension produced by acute bladder irritation by intravesicular acrolein (toxic metabolite) and 2) chronic bladder inflammation by multiple injections of intraperitoneal cyclophosphamide (toxic alkylating agent). In the acute bladder irritant model, animals were administered acrolein (0.4 mM, 100 µL) intravesically under light isoflurane anesthesia 1 hour before bladder distension was performed; the control group was pre-treated with intravesicular 0.9% saline. With the chronic bladder inflammation model,
animals received intraperitoneal injections of cyclophosphamide (80 mg/kg) at day 0, 2, 4, and 6 (Boudes et al., 2011). Control mice received i.p. injections of 0.9% saline. All experiments were conducted after the final day of CTX administration (day 7).

2.2.3 UBD Surgery. Wires for electromyographic recording were surgically implanted as described by Kamp et al. (2003) with minor modifications (Kamp et al., 2003). Animals were anesthetized with i.p. ketamine (27.5 mg/kg; Hospira, Lake Forest, IL) and xylazine (10 mg/kg; Bayer, Shawnee Mission, KS). The animals were then shaved to remove hair from the neck and the right side of the abdomen. The bare skin was disinfected with ethanol (50%) and iodine (0.70%) swabs. A surgical incision (1 cm in length) was made in the back of the neck and lateral to the abdominal midline. The external oblique abdominal musculature was identified and electrode wires (Teflon coated stainless steel wire; Cooner Wire Sales, Chatsworth, CA) were sewn just above the inguinal ligament. The wires were stripped to remove the plastic covering and placed 5 to 10 mm apart with surgical suture (6-0 Silk). The wires were then tunneled underneath the skin to emerge on the back of the neck and sewn to the neck muscles. A small loop (2 cm) of the wire was placed under the skin to allow the wire to accommodate to the natural movements of the animal and avoid loss of the wire after surgery. The neck and abdominal incisions were then closed using wound clips (Reflex 7 mm). To avoid fluid loss during surgery, animals were administered 0.1 mL of saline solution after completing the surgery. Also, a local topical anesthetic (lidocaine ointment) was applied to the incisions. Animals were single housed and allowed to recover a minimum of 72 hours. Mice that developed motor
defects, weight loss, or display stress associated behaviors were excluded from the experiments.

2.2.4 Catheterization of Bladder. Bladder distension was performed according to Ness et al. with minor modifications (Ness et al., 2004). Briefly, mice were anesthetized with 4% isoflurane (induction) and 1.5% (maintenance) for 5 min; (Halocarbon Laboratories, River Edge, NJ), and a PE 10 tubing lubricated with Surgilube (E. Fougera, Melville, NY) was inserted (5-8 mm) transuretherally into the urinary bladder. The catheters were kept in place by taping the PE tubing to the base of the tail, and secured to the urethral orifice with cyanoacrylate. Mice were placed in restraint devices (plastic semi-circular tubing) while still sedated and allowed to recover and acclimate for a minimum of 1 hour before testing. Verification of proper placement of catheter was done after euthanasia.

2.2.5 Restraint Devices. Restraint devices were made from 50 mL conical tubes. An opening (~7 X 9 mm) was made at the superior aspect of the tube for access to the EMG recording electrodes. Small 0.5 cm diameter holes were made at the proximal end of the tubes to facilitate breathing. Also, a 25% portion of the conical tube was removed horizontally and a rectangular glass (2 X 5 cm) was glued in its place to accommodate the animal to a flat surface and avoid rolling of the tube. The final restraining device holds a 17-27 gram mouse. After the mouse was placed in the tube, the distal (open) end was secured with a gauze square and paper tape. The tube was then placed in a dark-colored cotton infant sock to reduce ambient light. The animals were allowed to acclimate inside the tube for 60 minutes before beginning recordings. Animals inside the tube were monitored during experiments.
2.2.6 Bladder distension and EMG recordings. Visceromotor responses were quantified from the EMG signals due to urinary bladder distension evoked musculature contractions as previously described (Ness et al., 2004; Christianson & Gebhart, 2007). Graded intensities of urinary bladder distension (0.05, 0.1, 0.15, and 0.2 mL normal saline), at 2 minute intervals, were used to compare the differences between 1 week vehicle and 1 week ceftriaxone treated animals. Preliminary experiments determined that these volume distensions corresponded to 15, 30, 45 and 60 mm Hg, respectively. Although the EMG activity at the threshold micuturition pressure (~4 cm H2O in mice) was not recorded (Zhou et al, 2010), the recorded graded distension volumes may correspond to non-noxious and noxious distension pressures (Figures 2.2B, 2.4B, and 2.9B). EMG activity was recorded 10 seconds before bladder distension to establish baseline activity and 20 seconds during distension (response = increase above baseline). The EMG signal was then normalized as change over baseline using Spike2 data acquisition software.

2.2.7 Behavioral testing. Prior to beginning experiments animals were acclimatized to the cages in which observation of the animals occurred. After animals received 1 week CTX or vehicle treatments, on the day of the behavioral recordings animals were slightly anesthetized with isoflurane (1.5%) and a PE 10 tube was lubed with Astroglide ® jelly and placed inside the bladder via the urethra. A 1cc syringe containing either 0.9 % saline or acrolein (0.4 mM) was used to instill fluid (50 µL) into the bladder over 1 minute through the PE 10 catheter. Animals were placed in the observation chambers immediately after delivering fluid and allowed to acclimate for 30 minutes before initiating recording of time spent licking of abdominal area. All animals regained their
righting reflex before commencing study (>5 minutes), studies were conducted by blinded observers and the treated groups were randomized.

2.2.8 Western blot. To determine whether increased GLT-1 expression occurred after 1 week ceftriaxone compared to 1 week vehicle, mice were euthanized after these treatments and the lumbosacral region (L5 to S2) was excised. The spinal cord was homogenized using a hand-held pestle in a lysis buffer containing a commercial mixture of phosphatase inhibitors and proteinase inhibitors. The samples (40 µL; 80 µg) were loaded onto wells and run in a 10% SDS-PAGE gel, and then electroblotted onto nitrocellulose membrane using a minigel and mini transblot apparatus (BioRad laboratories, 170-3935). The membranes were then rinsed and placed in blocking buffer (20 mM Tris-HCl, pH=7.5, 137 mM NaCl, 0.1% Tween 20, and 15% nonfat milk) at room temperature for 1 hr. The membranes were then incubated with primary antibodies consisting of primary rabbit anti-EAAT2 (1:3,000; Santa Cruz) C-terminal antibody or the primary rabbit antibody β-actin (1:1,000; Santa Cruz) in 1% milk-PBST buffer overnight at 4°C. Afterwards, the membranes were exposed to the secondary antibody goat anti-rabbit IgG with horseradish peroxidase (HRP) in 1% milk-PBST buffer (1:6,000 dilution) for 1.5 hours. LumiGlo chemiluminescent substrates were used to detect HRP-antibody signal with X-ray film exposure. The optical densities of protein blots were analyzed by standardizing the optical density of EAAT2 against the optical density of the β-actin protein.

2.2.9 Glutamate uptake assay. Lumbosacral sections (L5-S2) of spinal cord were dissected from animals and minced into small pieces. Tissue from each mouse was
homogenized 10 strokes by a rotor-driven homogenizer in 3 mL ice-cold tissue buffer containing 1X complete proteinase inhibitor (Roche, lot. 11714800), 0.32 M sucrose and 0.05 M Tris (pH=7.4). Cell debris was removed by 1,000 g centrifugation for 10 min at 4°C, and the synaptosomal fraction in the supernatant was separated into 2 tubes, one with 2 mL and the other one with 1 mL of tissue buffer. Synaptosomes were then spun down at 16,000 g for 10 min at 4°C. For each animal, uptake was measured in triplicate: control group, dihydrokainic acid (DHK; GLT-1 antagonist) treated group and sodium-free control group. Synaptosomes were re-suspended in 1.5 mL Krebs buffer (pH=7.4) for first two groups, or in 1.5 mL Na⁺-free Krebs buffer (Cholone-Cl 120 mM, KCl 5 mM, MgSO₄ 1 mM, KH₂PO₄ 1 mM, Tris-Cl 25 mM, D-Glucose 0.55 mM, CaCl₂ 2 mM, pH=7.4). 200 μL of synaptosome suspension was loaded into clean eppendorf tubes and pre-incubated at 37°C for 10 min. 1 mM DHK was added into the DHK group. To initiate the reaction, 0.1 mM unlabeled glutamate and 0.05 μM tritium-labeled glutamate (Perkin Elmer Inc, USA, 1 mCi/mL) were added into each tube and tubes were incubated at 37°C for 10 min. 500 μL of ice-cold tissue buffer was added to stop the reaction and tubes were placed on ice. Synaptosome suspension was exposed to vacuum filtration by using filter paper pre-coated in 0.2% polyethylene solution. Synaptosomes retained by the filter paper were then washed three times by 4 mL PBS buffer and the filter paper was transferred to scintillation vials with 3 mL scintillation buffer and 0.1N NaOH. A filter paper that contained the 0.05 μM tritium-labeled glutamate without synaptosome incubation was used as negative control. Tritium labeled glutamate inside the synaptosome was measured in scintillation counter (Beckmann, USA). GLT-1 mediated
glutamate uptake is calculated by subtracting the uptake in the dihydrokainic acid-treated group from the normal control group. Sodium dependent glutamate uptake is calculated by subtracting the uptake in the Na-free negative group from the normal control group.  

2.2.10 Statistical Analysis. The student t-test was used to determine significant differences in the glutamate uptake assay. All elicited EMG signal were rectified and the area under the curve (AUC) for the 20 second distension was subtracted from the AUC of the 10 second baseline, as previously described (Ness et al., 2004). The quantitative data in each study was expressed as mean ± SEM. A random block design with a one way ANOVA was used for behavioral experiments. Analysis of variance was followed by Least Significant Difference (LSD) multiple comparison post-hoc tests; data were considered statistically significant different if p<0.05.

2.3 Results

2.3.1 Enhancement of GLT-1 expression and glutamate uptake activity blunts the visceromotor response to bladder distension

GLT-1 expression was enhanced 20% and glutamate uptake activity enhanced 113% in lumbosacral spinal cord after 1 week (1-wk) ceftriaxone (CTX; 200 mg/kg daily) treatment (Figure 2.1 A-B). The visceromotor response to bladder distension was compared in 1-wk vehicle versus 1-wk CTX treated animals (Figure 2.2 A). 1-wk CTX treatment resulted in a 60-64% decrease in the visceromotor response at each of the three highest distension volumes (0.1, 0.15, and 0.2 mL; *p<0.05). No significant difference
was observed at the lowest distension volume of 0.05 mL. A representative depiction of the blunted visceromotor response caused by 1-wk CTX is presented in Figure 2.2 B. Thus, similar to previous findings studying murine colo-rectal distension after GLT-1 over-expression (Lin et al., 2009), bladder distension-induced visceromotor response was also mitigated by GLT-1 over-expression.

**Figure 2.1.** A-B. CTX induced GLT-1 over-expression and increased glutamate uptake. A. Comparison of GLT-1 expression in LS spinal cord of mice administered 1 week vehicle (n=3) or CTX (200 mg/kg, daily; n=3). *p<0.05  B. Glutamate uptake activity comparison from LS spinal cord of mice administered 1 week vehicle (n=7) or CTX (200 mg/kg, daily; n=7). *p<0.05
Figure 2.2. A-B. Ceftriaxone treatment decreased VMR to UBD. A. The visceromotor response to graded bladder distension in female mice treated with 1-wk CTX (200 mg/kg, daily) were compared to mice treated with 1-wk vehicle. CTX treated animals showed a diminished visceromotor response compared to littermates treated with vehicle. *p<0.05. B. Raw representative electromyographic recordings elicited after graded bladder distension in 1-wk vehicle versus 1-wk CTX treated female mice.
2.3.2 Selective GLT-1 antagonist dihydrokainate reverses 1 wk ceftriaxone-induced mitigation of visceromotor response caused by bladder distension

To verify that 1 week CTX induced a decrease in the visceromotor response due to increased expression of GLT-1, a selective antagonist of the GLT-1 transporter, dihydrokainate acid was used to assess whether enhanced GLT-1 activity might mediate the diminished visceromotor response produced by 1 week CTX treatment.

Figure 2.3. The effect of systemic injection of selective GLT-1 antagonist dihydrokainate (DHK) on GLT-1 over-expression. One hour prior to graded bladder distension, 1-wk vehicle and 1-wk CTX group was treated with dihyrokainate (DHK) (10 mg/kg, i.p.). 1-wk CTX + vehicle produced a significantly reduced visceromotor response to bladder distension compared to the 1-wk VEH + VEH group at 0.1 and 0.15 mL volumes. *p<0.01
Dihydrokainate (10 mg/kg) was administered one hour before urinary bladder distension in animals treated with either 1 week vehicle or CTX. One-week CTX treated cohorts showed a (62-73%) reduction in the visceromotor response to graded bladder distension (Figure 2.3). Dihydrokainate pretreatment reversed the CTX-mediated significant reduction in visceromotor response to bladder distension (Figure 2.3).

2.3.3 Effect of GLT-1 over-expression on acrolein-induced bladder irritation changes in the visceromotor response to bladder distension

The effect of GLT-1 over-expression on local irritant-enhanced visceromotor response to bladder distension was studied. Intravesicular acrolein, administered 24 hours before the measurement of the visceromotor response to bladder distension produced a 30-83% increase in the response (Figure 2.4 A-B). GLT-1 over-expression produced by daily CTX treatment for 1 week completely attenuated this response. Thus, pharmacologic enhancement of glutamate uptake also reduced the enhancing effect of local bladder irritation on evoked visceral nociception.
Figure 2.4. A-B. The effect of GLT-1 over-expression on bladder irritant-sensitized visceromotor response. A. In animals treated with 1-wk vehicle and then intravesicular acrolein (●), a significant increased (30-83%) visceromotor response to UBD (*p<0.05) was observed, compared to intravesicular vehicle-treated mice (○). One-week CTX treatment abolishes the enhanced visceromotor response to bladder distension caused by intravesicular acrolein (▲). B. Raw representative electromyographic recordings.
2.3.4 Ceftriaxone dose ineffective to alter the normal visceromotor response to urinary bladder distension mitigates irritant-induced visceral hyperalgesia

Previous data studying visceral hyperalgesia showed that 1-wk CTX at a 200 mg/kg dose attenuates the normal visceromotor response to urinary bladder distension (Yang and Roman, 2011). Thus, the aim of this section was to study the effect of CTX treatments not affecting the normal visceromotor response to urinary bladder distension on hyperalgesia caused by a bladder irritant.

Figure 2.5. Lower doses of CTX treatment do not produce a VMR different from the control. Mice treated with 1-wk CTX (50 or 100 mg/kg) + ivc ACRO did not show an enhanced VMR to UBD response compare to 1-wk VEH + ivc VEH treated cohort at 0.15 and 0.2 mL. Animals that were administered 1-wk VEH + ivc ACRO showed a significant increase in VMR compared to the control group at 0.15 and 0.2 mL. *p<0.05
Animals received daily systemic injection of either 100 mg/kg or 50 mg/kg of CTX for 1 week, then 1 hour before graded urinary bladder distension, bladder irritation was induced via intravesicular (ivc) infused acrolein (0.4 mM). Cohorts treated with 1-wk VEH + ivc ACRO showed a significant increase (70-90% at 0.15 and 0.2 mL; *p<0.05) in VMR compared to 1-wk VEH + ivc VEH (Figure 2.5). CTX at doses not affecting the normal VMR response (50 and 100 mg/kg for 1 week) attenuated the visceral hyperalgesia caused by intravesicular acrolein at 0.15 and 0.2 mL (Figure 2.5).

2.3.5 Time spent licking of abdominal area after acute bladder irritation is reduced after 1-wk ceftriaxone

To confirm that ceftriaxone administration reverses visceral pain-like behavior in rodents, a quantifiable nociceptive response in unrestrained mice was elicited. In 1-wk vehicle or CTX (100 mg/kg) treated cohorts, the bladder was infused (50 µL) with either acrolein (0.4 mM) or 0.9% saline over a 1 minute period under light isoflurane anesthesia. After a 30 minute recovery time, the number of abdominal licks were quantified for 1 hr. Time spent licking was increased 180 % in the 1-wk VEH + ivc ACRO, cohort compared to control ( 1-wk VEH + ivc VEH). In contrast, animals treated with 1-wk CTX + ivc ACRO did not show an enhanced time spent licking of the abdomen compared to 1-wk VEH + ivc VEH (Figure 2.6).
Figure 2.6. Licking behavior increased after acrolein bladder infusion. The data demonstrates an increase in time spent licking in animals that received 1-wk VEH + ivc ACRO, compared to the control group (*p<0.05). This enhanced pain-like behavioral response was attenuated in cohorts receiving 1-wk CTX + ivc ACRO treatment. Experiment was conducted by observers blinded to treatment.

2.3.6 Effect of ceftriaxone on chronic bladder inflammation-induced changes in the visceromotor response to bladder distension

Previous studies on rodents showed that repeated injections of cyclophosphamide (CYP) lead to prolonged bladder irritation and inflammation and has been proposed as an animal model for interstitial cystitis (Vizzard et al., 1996; Bon et al., 2003; Wantuch et al., 2007). To further explore if CTX produces a reduction of viscero-motor response to bladder distension in this model, mice received one week CTX (200 mg/kg) treatment at the onset of induced inflammation of the bladder (at day 0) elicited by i.p. injection of CYP (80 mg/kg; days 0, 2, 4 and 6). The results indicate that the 1-wk VEH + CYP
cohort showed an enhanced visceromotor response to UBD compared to 1-wk VEH + VEH treated group (66-120% at 0.15 and 0.2 mL) (Figure 2.7A). 1-wk CTX administered to CYP-treated animals blunted the enhanced VMR to UBD seen in the 1-wk VEH + CYP cohort (Figure 2.7). Thus, the data suggest that GLT-1 over-expression by 1-wk CTX attenuated bladder inflammation-induced hyperalgesia.

Figure 2.7. High dose CTX treatment decreased VMR to UBD in mice with visceral hypersensitivity. The enhanced visceromotor response produced in the 1-wk VEH + CYP group was attenuated by 1-wk CTX administration (1-wk CTX + 1-wk CYP). Cyclophosphamide (1-wk VEH + 1-wk CYP) administration significantly increased the visceromotor to urinary bladder distension at 0.15 and 0.2 mL, compared to 1-wk vehicle + 1-wk vehicle treated mice. *p<0.05
A lower dose of CTX (100 mg/kg; i.p.) for seven days beginning at day 0 did not elicit a visceromotor response different from 1-wk VEH + 1-wk VEH treated cohorts after graded urinary bladder distension. The 1-wk VEH + 1-wk CYP group produced a significantly enhanced VMR to UBD (178-239% at 0.15 and 0.2 mL). However, mice treated with 1-wk CTX (100 mg/kg) + CYP (80 mg/kg; i.p.) showed no significantly enhanced visceromotor response (*p<0.05) compared to 1-wk VEH + 1-wk VEH controls (Figure 2.8).

Figure 2.8. Low dose CTX treatment decreased VMR to UBD in mice with visceral hypersensitivity. Administration of CTX (100 mg/kg; i.p.) for seven days did not alter the VMR from that of the control group at graded UBD. Moreover, cyclophosphamide treatment significantly increased the viscero-motor to urinary bladder distension at 0.15 and 0.2 mL, compared to 1-wk vehicle + 1-wk vehicle treated mice. *p<0.05
2.3.8 Cross organ sensitization: Effect of increased GLT-1 expression on sensitization of bladder nociception

Emerging evidence suggests that colon irritation can alter neuronal activity of afferents serving other visceral organs, such as the bladder (Malykhina et al., 2006; Pezzone et al., 2005; Ustinova et al., 2006). To determine if increased expression of GLT-1 can attenuate cross-organ sensitization resulting in enhanced visceromotor response to bladder distension, intracolonic TNBS (35 mg/mL; 100 µL) was administered 1 hour before the visceromotor response to graded bladder distension volumes was elicited in 1 week CTX and 1 week vehicle treated cohorts. Mice receiving 1 week VEH+ intracolonic TNBS had a 75 to 138% increase in the visceromotor response to graded bladder distension compared to animals receiving 1-wk VEH + intracolonic VEH (Figure 2.10 A-B). In marked contrast, animals treated with 1-wk CTX + intracolonic TNBS showed no enhanced visceromotor response compared to the 1-wk VEH + intracolonic VEH group. The data suggest that increased glutamate uptake via enhanced expression of GLT-1 by 1-wk ceftriaxone attenuated sensitized visceromotor response to bladder distension produced by colonic irritation.
Figure 2.9. A-B. CTX treatment decreases TNBS-induced cross-organ sensitization. A. Animals administered (1-wk) vehicle and intracolonic TNBS (●) showed a significant increase in the VMR to bladder distension (*p<0.05), compared to intracolonic vehicle-treated mice (○). Moreover, CTX treatment abolished the enhanced visceromotor response to UBD caused by intracolonic TNBS (▲). B. Raw representative electromyographic recordings elicited after graded bladder distension in different treatment cohorts.
2.4 Discussion

The principal findings of this study were that enhanced glutamate uptake by pharmacologically augmented GLT-1 expression 1) reduced the visceromotor response to urinary bladder distension by 2) effects reversible by the systemic injection of selective GLT-1 antagonist dihydrokainate (DHK), 3) enhanced visceromotor response caused by acute bladder irritation or bladder inflammation were significantly attenuated by enhanced glutamate uptake at high and low dose CTX and 4) cross organ sensitization (colon to bladder) of bladder visceromotor response to distension was also reduced by enhanced glutamate uptake. These findings expand on earlier observations showing that GLT-1 over-expression attenuates the visceromotor response to colo-rectal distension (Lin et al., 2009; Lin et al., 2011). Also, recent reports showing that gene therapy (Maeda et al., 2008) and pharmacologic (Hu et al., 2009) approaches to enhance GLT-1 expression reduce somatic pain suggest that strategies to reduce extracellular glutamate may provide new avenues for pain therapeutics.

Several studies have disclosed that the mechanisms of visceral and somatic pain have points in common and divergence (Dunckley et al., 2005; Sikandar et al., 2011). Among the similarities is the role of glutamate receptor-mediated activation of second order spinal neurons. Noxious stimuli evoke long-term increases in the excitability of dorsal horn neurons; glutamate, acting primarily at N-methyl-D-aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, contributes to the development of this phenomenon (McRoberts et al., 2001). NMDA and AMPA

63
antagonists also attenuate the visceromotor response to colonic distension (Al-Chaer et al., 1996; Traub et al., 2002). However, these agents have severe limitations as therapeutic agents due to the wide distribution of glutamate receptors throughout the central nervous system.

The over-expression of GLT-1 after systemic ceftriaxone is well characterized to occur preferentially in astrocytes found in the spinal cord and supraspinal regions (Rothstein et al., 2005). However, the anatomical site(s) of the beneficial effect of GLT-1 over-expression is unclear. A recent study using a rat neuropathic pain model revealed that intrathecal ceftriaxone was effective as both a preventative or therapeutic approach to relieve thermal hyperalgesia and to a lesser extent mechanical allodynia (Hu et al., 2009). Also, adenoviral-mediated spinal gene delivery of GLT-1 produced anti-nociceptive effects (Maeda et al., 2008). Moreover, transgenic animals or 1-wk ceftriaxone-treated animals over-expressing GLT-1 show no motor or behavioral abnormalities, and no changes in respiratory function (Guo et al., 2003; Rothstein et al., 2005). Enhanced glutamate uptake activity in spinal sites involved in visceral nociception is correlated with attenuated visceromotor response in this dissertation (Figure 2.1B; Figure 2.2A). Thus, the spinal cord, the first central synapse in the nociceptive pathway, is one likely site of action of enhanced GLT-1 expression to mitigate visceral pain (see chapter 3).

In addition, alteration in the visceromotor response to bladder distension caused by local bladder irritation was attenuated by GLT-1 over-expression at a high dose (200 mg/kg)(Figure 2.4 A-B) and low dose (100 mg/kg) (Figure 2.5). Thus, the strategy of reducing extracellular glutamate by GLT-1 over-expression appears effective to mitigate
enhanced bladder nociception due to locally activated bladder sensory mechanisms (Guo et al., 2006). An additional animal model of visceral nociception was used and demonstrated that intraperitoneal injections CTX (100 mg/kg) reduced abdominal licking response after acute bladder irritation (Figure 2.6). Suprisingly, the amount spent licking after (0.4 mM) acrolein infusion to the bladder shows a similar trend to that of the visceromotor response after the same concentration of acrolein is administered to the bladder. This suggests that the time spent licking after noxious stimulation to a visceral organ is a viable method to assess mice visceral nociception. To verify that the amount spent licking is a nociceptive response, morphine at graded concentrations should show a dose-dependent attenuation of the licking behavior due to bladder inflammation. Future studies should include other behavior parameters that may not be obvious after the infusion of acrolein to the bladder, such as lack of movement.

Moreover, studies conducted in mice showed that repeated intraperitoneal injections of cyclophosphamide, which is metabolized into acrolein and is concentrated in the bladder, induces prolonged (chronic) bladder inflammation that models aspects of interstitial cystitis (Boudes et al., 2011; Lai et al., 2011). The results of this study show that GLT-1 over-expression via 1-wk CTX diminished the enhanced visceromotor response to bladder distension caused by chronic bladder irritation (Figure 2.7). Moreover, 1-wk CTX administration at a lower dose (100 mg/kg) was effective in mitigating the enhanced visceromotor response after bladder inflammation via CYP treatment, without altering the baseline visceromotor response (Figure 2.8). Thus, a high (200 mg/kg) and low (100 mg/kg) dose of 1-wk ceftriaxone treatment at the onset of
bladder inflammation attenuates the enhanced VMR cause by bladder inflammation.

Similar results were observed in animals treated with low dose 1-wk ceftriaxone treatment and received acute bladder irritation (Figure 2.5). This is an important step toward future clinical studies on the potential anti-nociceptive therapeutic effect of ceftriaxone, which has been proven not to alter locomotion or memory functions (Miller et al., 2008; Lin et al., 2011).

Further findings of this study revealed that 1) acute colon irritation produced enhanced visceromotor response to bladder distension (colon to bladder sensitization) and 2) colon to bladder cross organ sensitization was attenuated by pre-emptive GLT-1 over-expression (Figure 2.9 A-B). Multiple mechanisms have been advanced to explain cross-organ sensitization; these mechanisms can be conveniently categorized as either central or peripheral (Gebhart et al., 2010). Irritant-induced sensitization of afferent nerve endings (peripheral and/or central) serving multiple visceral organs, or development of central sensitization within second order neurons or higher in the neuroaxis are among proposed mechanisms of cross-organ sensitization (Qin et al., 2005). Given the importance of glutamate as the predominant excitatory neurotransmitter of visceral afferents (Talman et al., 1980; Cotman and Iversen, 1987), the present report suggests that reduction of extracellular glutamate significantly reduces glutamatergic transmission mediating cross-organ sensitization. Moreover, the findings of the present study suggest that attenuation of glutamatergic transmission mitigates colon irritation-enhanced visceromotor response to bladder distension (Figure 2.9 A-B).
In contrast to the effect of dihydrokainate on ceftriaxone-blunted visceromotor response to colon distension (Lin et al., 2009), the reversal of the effect of 1-wk ceftriaxone by dihydrokainate treatment was not complete at the 0.1 and 0.15 mL distension volumes (Figure 2.3 A). Potential explanations include: 1) the optimal time of dihydrokainate administration was missed with regard to reversal of ceftriaxone- mitigation of the visceromotor response to bladder distension, 2) response variability due to different phases of the estrus cycle present within the cohorts or 3) other effects produced by ceftriaxone (i.e., effects on gastrointestinal flora) (Verdue et al., 2006) may mediate its reduction of the visceromotor response to bladder distension.

The exact mechanism of GLT-1 over-expression responsible for the blunted visceral pain response awaits further exploration. However, reduced activation of spinal glutamate receptors in GLT-1 over-expressing animals during urinary bladder distension that results in a reduced activation of second order spinal neurons, is a leading possibility. Decreased activation of second messenger pathways (PKA, PKC, PIP2, NO/GC/PKC, ERK, p38) downstream from glutamate receptors also may be involved (Sakurai et al., 2008).

This report extends work showing the effectiveness of enhanced glutamate uptake to mitigate the visceromotor response to bladder distension, bladder irritation or inflammation models, and colon to bladder cross-organ sensitization. An important unexplored question surrounds the therapeutic application of this approach (i.e., does GLT-1 upregulation mitigate previously established cross-organ sensitization of bladder nociception and function). A recent study suggests therapeutic effectiveness of GLT-1
over-expression in an animal model of colitis after receiving dextran sulfate sodium (Lin et al., 2011). Although the present study demonstrated that GLT-1 over-expression significantly attenuated chronic visceral nociception, the long-term therapeutic benefits of GLT-1 over-expression in an animal model of cystitis need to be addressed. Success of this approach fuels optimism that strategies to enhance GLT-1 activity may lead to improved mechanistic-based therapeutic options for treating visceral hyperalgesic disorders.
Chapter 3

Characterization of anti-nociception effect of GLT-1 over-expression: site of action, GluR1 trafficking, and neonatal stress model

3.1 Introduction

Visceral pain disorders disrupt daily activity for a significant portion of the U.S. population and these conditions can lead to a poor quality of life. For example, interstitial cystitis/painful bladder syndrome (PBS) is associated with several symptoms that include changes in function of the bladder and inflammation leading to severe pain (Abdel-Mageed et al., 1998). Overall therapeutic options for visceral pain disorders are limited largely to symptomatic relief, without focus to the underlying etiology due to the complexity of the visceral sensory system (Erickson et al., 2005 and 2008; Spiegel et al., 2010). Pain-transmitting afferent neurons utilize glutamate as the principle neurotransmitter to signal nociception related to the visceral organs (Willert et al., 2004; Schicho et al., 2005). Therefore, a decrease in spinal extracellular glutamate may be important in pain management.

The spinal glutamatergic system plays a vital role in mediating nociception associated with the somatic and visceral systems (Yang et al., 1996; Willert et al., 2004).
Glutamate transporters are essential for maintaining homeostatic levels of extracellular glutamate (Kanai and Hediger, 2003). Previous studies show that inhibition of glutamate transporters in the spinal cord is responsible for inducing hyperalgesia and increases neuronal activity (Weng et al., 2006). Activation of glutamate transporters is effective to mitigate somatic nociception (Maeda et al., 2008; Hu et al., 2010). However, a role of glutamate transporter activation in modulating visceral nociception has not been well studied. Although it is known that 1) noxious stimuli to visceral tissue leads to central sensitization of visceral afferents (Zhou et al., 2009) and 2) the activation of glutamate receptors mediates central sensitization (Cervero et al., 1995; Peles et al., 2004), the putative role of glutamate transporter upregulation to mitigate visceral nociception has not been fully explored.

Spinal glutamate receptors found post-synaptically enhance the activity of second order pain-transmitting neurons by modulating the influx of calcium (Mayer et al., 1985; Mayer et al., 1987; Zuromboski et al., 1989). The influx of cations to the post-synatpic terminals, due to ionotropic glutamate receptors NMDA and AMPA, can activate signaling pathways that generate central sensitization (Li et al., 1999; Morrisey et al., 2000; Fang et al., 2002). The ERK1/2 signaling pathway is important for the development of central sensitization and it is modulated by glutamate binding to glutamate receptors (Hu et al., 2007). Studies have revealed that the glutamate receptor NMDA is involved in activation of c-fos and ERK1/2 pathway after inflammation of the colon (Eijelkamp et al., 2007; Qiao et al., 2008). Similarly, inflammation and distension
of the bladder increases the expression of ERK1/2 and phosphorylation of ERK 1/2 in the lumbosacral region of the spinal cord (Lai et al., 2011).

Evidence suggests other glutamate receptors mediate visceral sensation and hyperalgesia. Enhanced trafficking of the AMPA receptor subunit GluR1 to the cell membrane has been observed after intracolonic capsaicin (Galan et al., 2004). Moreover, studies suggest that a similar event occurs in animals models of inflammatory pain (Harmann et al., 2004; Kopach et al., 2011). Hence, the role of GluR1 trafficking during visceral sensitization may help explain the diminished visceral nociceptive response in animals that over-express glutamate transporters and reduce extracellular glutamate.

Dihydrokainte acide (DHK) is a selective inhibitor of the glutamate transporter GLT-1 due to its similar structure to glutamate and the potent inhibitory components of DHK are found in the glutamate conformer that contains isopropyl groups in four positions (Arriza et al., 1994). Therefore, DHK has been used to study the the function of the GLT-1 under several neurotoxic conditions. Previous studies showed that systemic or intrathecal injection of DHK, reverses the anti-nociceptive effect of GLT-1 over-expression after colo-rectal distension (Lin et al., 2011).

Thus, the first goal of this chapter was to determine whether the spinal cord mediates the effect of GLT-1 over-expression to attenuate the visceromotor response observed in animals that undergo urinary bladder distension. The second goal of this chapter was to establish whether enhanced trafficking of the GluR1 subunit mediates the pro-nociceptive effects of chronic visceral inflammation. These studies were designed to determine if GLT-1 over-expression can mitigate trafficking of the GluR1 subunit to the
post synaptic membrane after persistent inflammation of the bladder, a model of interstitial cystitis. The third goal of this chapter was to establish if GLT-1 over-expression suppress hyperalgesia, to colonic distension due to early life stress, an animal model of irritable bowel disease. The final goal of this chapter was to study the duration of the anti-nociceptive effect of 1 week CTX (200 mg/kg) to urinary bladder distension.

3.2 Methods

3.2.1 Animals. Two month old female FVB/N mice (20-25g) were used for the experiments. Animals were housed singly on wood shavings with ad libitum access to food and water. The 12 hour light-dark cycle was maintained. Prior to experiments animals were fasted for 12 hours to limit the fecal content of the colon; ad libitum access to water was maintained. Experiments were approved by Institutional Animal Care and Use Committee from The Ohio State University and followed the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

3.2.2 Drug Administration. A high dose (200 mg/kg) or low dose (100 mg/kg or 50 mg/kg) of ceftriaxone was prepared in 0.9% saline and administered intraperitoneally (i.p.) at 10:00 am for seven consecutive days (1 week); the control group received i.p. 0.9% saline injections. In some experiments, the selective GLT-1 antagonist dihydrokainate was administered (10 µL) at a concentration of 0.3 mM, 0.03 mM, or 0.003 mM intrathecally (i.t.) one hour prior to urinary bladder distension; using a 28G
needle attached to a 10 µL Hamilton syringe the control group received 0.9% saline i.t. injections.

3.2.3 Urinary bladder distension surgery. As previously described in chapter 2, wires for electromyographic recording were surgically implanted in the external oblique muscle of mice. Briefly, animals were anesthetized with i.p. ketamine (27.5 mg/kg; Hospira, Lake Forest, IL) and xylazine (10 mg/kg; Bayer, Shawnee Mission, KS) mice were shaven at the neck and the right side of the abdomen, the incision areas were disinfected, and a surgical incision (1 cm in length) was made in the back of the neck and lateral to the abdominal midline to expose the external oblique abdominal musculature. Then the electrode wires (Teflon coated stainless steel wire; Cooner Wire Sales, Chatsworth, CA) were sewn just above the inguinal ligament. The wires were then tunneled underneath the skin until they protruded on the back of the neck and sewn to the neck muscles. The neck and abdominal incisions are then closed using wound clips (Reflex 7 mm). To avoid fluid loss during surgery, animals were administered 0.1 mL of saline solution after completing the surgery. Animals were single housed and allowed to recover a minimum of 72 hours. Mice that developed motor defects, weight loss, or display stress associated behaviors were excluded from the experiments.

3.2.4 Catheterization of Bladder. Bladder distension was performed according to Ness and Elhefani (2004) and as previously described in chapter 2 (Ness and Elhefani, 2004). On day of UBD experiment, mice were anesthesized with 4% isoflurane (induction) and 1.5% (maintenance) (Halocarbon Laboratories, River Edge, NJ), and a PE 10 tubing lubricated with Surgilube (E. Fougera, Melville, NY) was inserted (5-8 mm)
transuretherally into the urinary bladder. The catheters were kept in place by taping the PE tubing to the base of the tail, and secured to the urethral orifice with cyanoacrylate. Mice were placed in restraint devices (plastic semi-circular tubing) while still sedated and allowed to recover and acclimate for a minimum of 1 hour before testing. Verification of proper placement of catheter was done after euthanasia.

3.2.5 Restraint Devices construction. Restraint devices were made from 50 mL conical tubes. An opening (~ 9 mm in diameter) was made on top of the tube to access the EMG recording electrodes protruding from the neck of the animal (see section 3.2.3). Another small circular hole, 0.5 cm in diameter, was made at the proximal end of the tubes to facilitate breathing. In addition, two longitudinal cuts (2 cm apart) were made on the conical tube to remove the plastic (not containing breathing holes) and a rectangular glass (2 cm X 5 cm) was glued in its place to accommodate the animal to a flat surface and avoid rolling of the tube. The final restraining device holds a 17-27 g mouse. After the mouse was placed in the tube, the distal (open) end was secured with a gauze square and paper tape. The tube was then placed in a dark-colored cotton infant sock to reduce ambient light. The animals were allowed to acclimate inside the tube for 60 minutes before beginning recordings. The behavior of the mice before, during, and after distension was easily monitored by partial retraction of the fabric.

3.2.6 Bladder distension and EMG recordings. Visceromotor responses were quantified from the electromyographic (EMG) signals due to urinary bladder distension evoked musculature contractions as previously described in chapter 2. Briefly, graded intensities of urinary bladder distension (0.05, 0.1, 0.15, and 0.2 mL normal saline) were used to
compare the differences in the VMR between 1 week vehicle and 1 week ceftriaxone treated animals. Preliminary experiments determined that these volume distensions corresponded to (15, 30, 45 and 60 mm Hg) pressure, respectively. Electromyographic activity generated from the external oblique muscle, (visceromotor responses), were recorded 10 seconds before bladder distension to establish baseline activity and 20 seconds during distension (response = increase above baseline), and 10 seconds after termination bladder distension. The EMG signal was quantified (area under the curver) by measurement of the response during UBD, minus the 10 seconds preceding UBD (baseline) using Spike2 data acquisition software.

3.2.7 Neonatal colonic irritation. The following experiment was conducted as previously described by Christianson et al. (2010) with slight modifications (Christianson et al., 2010). A 26 gauge microfil needle (World precision Instruments, Inc) was attached to a 50 µL Hamilton syringe and either 10 µL of 2 % mustard oil (Sigma, St. Louis, MO) or 0.9% saline was delivered intracolonically (0.2 cm) on day 9 and 11 of age. To avoid damaging/sensitization of the surrounding tissue, Astroglide® was applied liberally to the perianal region. Animals were observed briefly (<5 min) for any damage to the tissue prior to being returned to the dam. All pups remained with dams until weaning on postnatal day 21. Prior to undergoing colo-rectal distension, 8-week old mice received either 1 week CTX (200 mg/kg) or 1 week vehicle (0.9% saline) treatment.

3.2.9 Colo-rectal distension balloon. Although Kamp et al. (2003) described the first assembly of colo-rectal distension balloons in rodents, the detailed protocol published by Christianson and Gebhart (2007) was used to construct the balloons with slight
modifications (Kamp et al., 2003; Christianson and Gebhart, 2007). A polyethylene tube (PE-60) was cut 10 cm in length and one end of the tube (1 cm) was punctured with a 27G needle to improve air flow into the balloon. The flat end of a rod (1 cm X 1 cm) was used to stretch a square polyethylene plastic (5 cm X 5 cm) by pressing against the flat end of the rod until the polyethylene plastic resembled a ~2.5 cm long cylinder. The PE-60 tube (containing the air holes) was inserted 1.5 cm inside the stretched polyethylene plastic and the open portion of the plastic was tightly sealed by a suture silk knot of the plastic and PE-60 tube. Excess thread and plastic protruding from the knotted site of the balloon was trimmed and a small drop of glue was placed on the knot to minimize leakage of the balloon. To determine the proper placement of the balloon in the distal colon the PE-60 tube was marked at 0.5 cm (corresponding to the location of the anus) and 1.5 cm (check point during experiment) from the knot. To protect the balloon from folding during rectal insertion a PE-240 tube (4 cm long with longitudinal cut) was used as a cover for the balloon and was removed after the balloon was secured in place (based on markers).

3.2.10 Colo-rectal distension experiment. Colorectal distension was performed as previously described by Kamp et al. (2003) with minor modifications (Kamp et al., 2003). Animals were anesthetized with 4% isoflurane (adjusted to 1.5% for most of the procedure). A lubricated sheath (PE-240 tube; 4 cm long) snugly covering a balloon attached to a PE-60 tube (10 cm) was inserted into the distal colon (2.5 cm) and the protective sheath was removed. The balloon was secured in place by taping the exposed PE-60 tube to the base of the tail. The EMG electrodes protruding from the neck and
protected with a Teflon coating were stripped to expose wire filaments. The animal was placed in a restraining device as previously described in section 3.2.5. The electrodes were attached to an amplifier connected to a CED recording apparatus (see section 3.2.6) and animals were allowed to recover and acclimate for 1 hr before commencing CRD. The visceromotor response to colorectal distension was elicited by intracolonic balloon distension at 15, 30, 45, and 60 mm Hg (10 seconds of baseline recording; 40 seconds of response), each distension pressure was repeated three times with 3 minute intervals. Once the CRD experiment ended the position of the balloon (0.5 cm beyond the end of balloon, marked) was checked and the balloon was withdrawn from the colon to test for air leakage. Also, animals were examined for signs of rectal bleeding due to injury caused by insertion of the balloon and excluded from the study. Data was analyzed as the number of EMG spikes above baseline with Spike2 software (Cambridge Electronic Design, Cambridge, UK).

3.2.11 Spinal cord extraction and fractionation of proteins. The fractionation of proteins was done as previously described by Park et al. (2008) with minor modifications (Park et al., 2008). Mice were euthanized by decapitation and the lumbosacral region of the spinal cord was excised and placed in a 5 mL tube containing 600 µL of lysis buffer (10mM Tris, 250 mM sucrose, 2 mM EGTA, 1 mM PMSF, 1mM benzamidine, 2 mM leupetin, 1:100 proteinase inhibitor cocktail, pH=7.4) and homogenized via a tissue grinder (Wheaton, USA). The samples were centrifuged at 1,000 g for 15 minutes at 4°C. Afterwards, 20 % of the supernatant containing the total protein (S1) was place in a 1.5 mL tube and 80% of the remaining supernatant was centrifuged at 10,000 g for 20 min at 4°C.
The supernatant containing the cytosolic fraction (S2) was collected and the remaining pellet was lysed in dH$_2$O at 4°C and ultracentrifuged at 20,500 g for 30 minutes at 4°C. The supernatant was discarded and the remaining pellet containing the synaptosomal membrane was collected and dissolved in modified 1X lysis (from stock) buffer (10 mM Tris, 250 mM sucrose, 2 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 2 mM leupetin, 1:100 proteinase inhibitor cocktail, 2% SDS, 0.1 % Triton X 100, in 7.99 mL of dH$_2$O, pH=7.4). The pellet is then sonicated with five quick bursts. Protein concentration of S1, S2, and P3 was determined with a Comassie (Bradford) protein kit assay (Thermo Scientific).

3.2.12 Western blot. The western blot was conducted as previously described in Chapter 2 with slight modifications. The complete lumbosacral spinal cord was homogenized and protein concentration quantitated as described in section 3.2.11. The desired final protein concentration (60 µg) was loaded in 8% SDS-PAGE gel, and then electroblotted onto nitrocellulose membrane using a minigel and mini transblot apparatus (Bio-Rad laboratories, cat.170-3935). The membranes were then rinsed and blocked with 3% nonfat milk in TBST buffer (0.1% tween 20, 20 mM Tris, 137 mM NaCl; pH=7.6) at room temperature for 1 hour. The membranes were then incubated with the primary rabbit polyclonal anti-glutamate receptor 1 (1:1,000; Millipore; AB1504) in 3% milk-TBST buffer overnight at 4°C. Afterwards, the membranes were exposed to the secondary antibody goat anti-rabbit IgG with horseradish peroxidase (HRP) in 3% milk-TBST buffer (1:3,000 dilution) for 1.5 hours at room temperature. Amersham ECL western blotting reagents (GE Healthcare; lot. 4624547) were used to detect HRP-antibody signal. The nitrocellulose membrane was rinsed with stripping buffer (0.2 M...
glycine and 0.05% tween 20; pH=2.5) for 45 minutes, washed (TBST), and placed in blocking buffer (3% nonfat milk) at room temperature for 1 hour. Then relabeled with rabbit polyclonal anti-β-actin (1:1,000; Santa Cruz; sc-130656). The membranes were exposed to the secondary antibody goat anti-rabbit IgG with horseradish peroxidase (1:3,000 dilution; Bio-Rad; cat.170-6515) in 3% milk-TBST buffer for 1.5 hours. Amersham ECL western blotting reagents (GE Healthcare; lot. 4624547) were used to detect HRP-antibody signal.

3.2.13 Statistical Analysis. A random block design with a one way ANOVA used for experiments. Analysis of variance were followed by Least Significant Difference (LSD) multiple comparison post-hoc tests when appropriate. Bio-Rad Quantity One software was used to determine optical density of western blots. Moreover, the optical densities of protein blots were analyzed by standardizing the optical density of GluR1 against the optical density of the β-actin protein. The quantitative data in each study was expressed as mean ± SEM; data were considered statistically significant different if p<0.05.

3.3 Results

3.3.1 Attenuated visceromotor response to urinary bladder distension produced by 1 week ceftriaxone is reversed by intrathecal dihydrokainate

To confirm that activation of glutamate transporter GLT-1 mediates the effect of 1-wk CTX to attenuate bladder distension induced nociceptive response, a selective GLT-1 antagonist, dihydrokainate, was administered intrathecally (10 µL) at different
concentrations (0.3, 0.03, and 0.003 mM) to assess if GLT-1 over-expression at the spinal cord or dorsal root ganglion region is responsible for mediating the reduced visceromotor response to urinary bladder distension. The results show that animals treated with 1-wk CTX + i.t. VEH showed a significant 62-70% decreased in visceromotor response to 0.15 and 0.2 mL bladder distension volumes compared to animals treated with 1-wk VEH + i.t. VEH (Figure 3.1). The CTX-mitigated nociceptive response was reversed in animals pretreated with i.t. DHK (0.3 mM and 0.03 mM) 1 hour before the experiment (Figure 3.1 and Figure 3.2). The highest intrathecal DHK dose tested (1-wk VEH + i.t. DHK at 0.3 mM) produced a significant increased VMR (129-93 % at 0.15 and 0.2 mL) compared to the control group (1-wk VEH + i.t. VEH) (Figure 3.1).

Figure 3.1. Dihyrokainate reverses the effect of ceftriaxone. Intrathecal injection of DHK at 0.3 mM, a selective GLT-1 antagonist, reversed the attenuated VMR induced by 1-wk CTX (ns; no significance). Moreover, 1-wk CTX + intrathecal VEH showed an attenuated VMR to UBD. *p<0.05
To determine if DHK reversed the effect of GLT-1 over-expression at a dose not affecting control response (1-wk VEH + i.t. VEH), a ten-fold lower dose of DHK (0.03 mM) was administered intrathecally and the VMR to UBD recorded. The cohort treated with 1-wk CTX + i.t. DHK at 0.03 mM showed a reversal of the attenuated VMR produced in the 1-wk CTX + i.t. VEH cohort at 0.15 and 0.2 mL (Figure 3.2), and 1-wk VEH + i.t. DHK 0.03 mM treatment did not significantly alter the visceromotor response to graded UBD (p>0.05) compared to control (1-wk VEH + i.t. VEH) cohorts (Figure 3.2).

Figure 3.2. A lower concentration of dihyrokainate reverses the effect of ceftriaxone. DHK (0.03 mM) did not enhance the VMR to UBD in the 1-wk VEH + i.t. DHK treated group, compared to the control group (ns; no significance). In addition, the 1-wk CTX + i.t. VEH treatment had a diminished VMR to UBD. * p<0.05
Figure 3.3. Dihyrokainate at 0.003 mM does not reverse the effect of ceftriaxone.

Intrathecal injection of DHK at 0.003 mM does not reverse the 1-wk CTX effect at the lower concentration. Moreover, 1-wk CTX attenuated VMR to UBD. *p<0.05

Intrathecal administration of an additional ten-fold lower dose of i.t. DHK (0.003 mM) was ineffective to reverse the effect of 1-wk ceftriaxone on the visceromotor response to bladder distension of 0.15 and 0.2 mL (Figure 3.3). Intrathecal injection of selective GLT-1 antagonist DHK reversed the anti-nociceptive effects of 1 week ceftriaxone administration.

3.3.2 Altered GluR1 trafficking is involved in persistent bladder pain

Under persistent inflammatory conditions the AMPA subunit GluR1 is found in the post-synaptic membrane and allows Ca^{2+} permeability to the cytoplasm of neuronal cells; hence, trafficking of the GluR1 subunit is important for neuronal activity via
glutamate binding to AMPA (Figure 3.4). Previous studies showed that GluR1 trafficking to the cell membrane is enhanced after intracolonic instillation of capsaicin (Galan et al., 2004).

**Figure 3.4. AMPA subunit trafficking.** Schematic showing probable interaction of the astrocytic glutamate transporter GLT-1 and postsynaptic glutamate receptor AMPA and GluR1 subunit after chronic noxious stimuli to the bladder.

In the present study, the lumbosacral region of the spinal cord showed a 19.9% increase in total GluR1 expression after 1-wk VEH + cyclophosphamide treatment compare to control 1-wk VEH + VEH cohorts (Figure 3.5-3.6). This total increase in GluR1 expression after 1-wk VEH + cyclophosphamide was due to trafficking to the post-synaptic membrane; a 21.5% increase in GluR1 expression (Figure 3.53-3.6). However, GLT-1 over-expression after 1-wk CTX + cyclophosphamide prevents trafficking of GluR1 to the post-synaptic membrane (Figure 3.3A-B). Although GluR1 is present in the cytosol (Figure 3.5-3.6), there was no significant difference between any of the treated groups and the control 1-wk VEH+VEH cohort (Figure 3.5).
Figure 3.5. GluR1 trafficking is mitigated after GLT-1 over-expression. Mice treated with 1-wk VEH + CYP showed an overall increased expression of GluR1 and increased trafficking to the post-synaptic membrane compared to control cohorts 1-wk VEH + VEH.
Figure 3.6. GluR1 is increased in the membrane after cyclophosphamide treatment.

Western blot densitometry shows a 19.9% increase in total GluR1 expression in 1-wk VEH + CYP and a 21.5% increase in the membrane compared to control cohorts 1-wk VEH + VEH. *p<0.05

3.3.3 GLT-1 over-expression mitigates the visceromotor response in adult mice after neonatal intracolonic mustard oil administration

Studies show that early life injury or insult of visceral organs induces long-term visceral hypersensitivity that can be observed by colonic distension (Winston et al., 2007; Christianson et al., 2010). Data shows that animals that received 2% mustard oil (MO) on postnatal day 9 and 11 produced a significant enhanced VMR to CRD at 8 weeks of age at distension pressures 30, 45, and 60 mm Hg, compared to 1-wk VEH + inta-colonic VEH cohorts (Figure 3.7). A markedly diminished VMR to CRD is observed in mice that received 1-wk CTX + intra-colonic MO treatment at all distension pressures compare to 1-wk VEH + intra-colonic VEH cohort. Thus, therapeutic treatment with CTX was
quite effective at mitigating visceral hypersensitivity caused by noxious neonatal colonic insult.

Figure 3.7. Neonatal stress induced visceral hypersensitivity in adult mice. Postnatal day 9 and 11 treatment of 2% mustard oil (MO) significantly increased the VMR to CRD at 30-60 mm Hg (1-wk VEH + intra-colonic MO) in adult mice, compared to control cohorts. *p<0.05

3.3.4 The anti-nociceptive effect of 1 week CTX is diminished within 30 days

To establish the duration of the anti-nociceptive effect of 1 wk- ceftriaxone, animals were treated with systemic 1-wk CTX (200 mg/kg) and the VMR to UBD was quantified at graded bladder distension volumes. The results show that 1-wk CTX had no significant (p>0.05) effect in mitigating VMR to UBD 30 days after at any distension volume (Figure 3.8).
3.4 Discussion

These set of studies determined that reduced visceromotor response to urinary bladder distension as a result of enhanced glutamate transporter GLT-1 (discussed in chapter 2) was reversible by intrathecal administration of the selective GLT-1 antagonist dihydrokainate (DHK). Hence, the evidence suggests that GLT-1 over-expression attenuates the visceromotor response to bladder distension via a spinal or dorsal root ganglia pathway. Previous studies assessing colo-rectal distension showed similar results (Lin et al., 2009). Therefore, DHK dose-dependently reverses the effect of 1-wk CTX + VEH on the visceromotor response to urinary bladder distension and spinal GLT-1 over-expression may be a significant contributor to the afferent anti-nociceptive effect of ceftriaxone (Figure 3.1-3.3).
However, this work cannot dismiss the possibility that GLT-1 over-expression is still important in supraspinal regions or in peripheral neurons (dorsal root ganglia). Regarding potential peripheral sites of action, there are emerging data suggesting that DRG may be a site of action of glutamatergic mechanisms mediating the nociceptive response (Jasmine et al., 2010; Carozzi et al., 2011). Studies suggest that GLT-1 is found in the DRG cells (Carozzi et al., 2011), but whether 1-wk ceftriaxone results in its over-expression has not been addressed. Interestingly, the glutamate transporter GLAST is predominantly expressed in satellite cells and EAAC1 in DRG neurons (Berger and Hediger, 2000; Tao et al., 2004; Carozzi et al., 2011). Hence, the mechanism of action and a possible anti-nociceptive effect by CTX due to satellite cell increased glutamate transporter uptake activity is an intriguing possibility worthy of further exploration. Regarding possible supraspinal sites of action, activation of glutamate transporters in the locus coeruleus can reduce somatic hypersensitivity in rats (Hayashida et al., 2010). Further work will be necessary to ascertain possible contribution of supraspinal or peripheral systems in mediating the apparent anti-nociceptive actions of GLT-1 over-expression. Overall, the present data supports 1) the potential translational approach of glutamate transporter over-expression to reduce glutamatergic neurotransmission and mitigate nociception, 2) the spinal cord may be the site of action of GLT-1 over-expression to mitigate the visceral nociceptive response in both bladder and colon. As a result, pharmacologically enhanced GLT-1 expression may provide a strategy to reduce extracellular glutamate and mitigate visceral nociception.
Ionotropic and metabotropic glutamate receptors play an important role in modulating spinal neuronal activity. Studies have demonstrated the importance of NMDA receptors in modulating sensory information from the spinal cord to the supraspinal regions via Ca\(^{2+}\) dependent intracellular mechanisms (Ji et al., 1999; Morisset et al., 2000). The role of NMDA in central sensitization has been extensively studied (Woolf and Thompson, 1991; Chizh et al., 1997). Although NMDA receptors are crucial contributors in central sensitization, the importance of AMPA receptors in establishing central sensitization is receiving attention (Li et al., 1999; Fang et al., 2002; South et al., 2003; Katano et al., 2008). Since AMPA mediated cation influx in the spinal cord is regulated by glutamate binding (Gu et al., 1996) and the GluR1 subunits contributes to changes in calcium permeability (Zhou et al., 2009), changes in GluR1 trafficking become important in studies of chronic pain (Burnashev et al., 1992; Tao, 2010). Studies have demonstrated that enhanced GluR1 trafficking takes place in somatic inflammatory pain (Harmann et al., 2004; Luo et al., 2008; Tao, 2010) but this has not been examined in the context of prolonged visceral pain. Although Galan et al. (2004) showed that GluR1 trafficking is significantly enhanced after capsaicin administration to the colon, the model used was acute (Galan et al., 2004). The present study suggests that chronic bladder inflammation significantly increased GluR1 expression to the synaptic membrane by 21.5% (Figure 3.5-3.6). The small enhancement of GluR1 expression in the membrane suggests that prolonged bladder inflammation results in GluR1 trafficking to the membrane that does not subside and this sustained GluR1 trafficking may contribute to the maintenance of sensitization. One week CTX treatment diminished these modest
enhancements (total GluR1 and trafficking of GluR1 to the membrane) (Figure 3.5-3.6). The data supports the notion that over-expression of GLT-1 may contribute to mitigation of visceral nociception by limiting GluR1 trafficking that contribute to enhanced sensory signaling of second-order neurons. But it is unclear whether long term changes in other postsynaptic factors (e.g., MAP kinases and NMDA subunits) may contribute to the antinociceptive actions of CTX.

Previous studies have established the effect of GLT-1 over-expression via CTX on acute (Lin et al., 2009; Yang and Roman, 2011) and chronic models (Lin et al., 2011) of visceral pain. However, these studies were conducted in adult mice and do not address the importance of early life stress in the development of visceral pain states (Ren et al., 2007; O’Mahony et al., 2008; O’Mahony et al., 2009). Early life stress produces changes in the developing CNS that lead to adult hyperalgesia (Cameron et al., 2008). Previous studies showed that mice administered 2% mustard oil intracolonically at postnatal day 8 and 10 have an increased colonic hypersensitivity as adults (Christianson et al., 2008). Therefore, the effects of GLT-1 over-expression in adult mice that had undergone early life stress via noxious irritation to the colon with 2% mustard oil (MO) was studied. The data shows that cohorts that received 2% mustard oil irritation had a markedly increased VMR to CRD at all distension pressures (Figure 3.7). However, animals treated with mustard oil as neonates and that received 1-wk CTX treatment as adults showed a dramatic attenuation of visceral hypersensitivity. Therefore, GLT-1 over-expression shows promise as a viable treatment for visceral nociception.
The next set of studies explored the duration of the beneficial effects of 1wk CTX to mitigate visceral nociception. A study conducted in the nucleus accumbens in rats showed that 5 day CTX treatment at doses 200 mg/kg and 100 mg/kg enhances GLT-1 glutamate uptake activity until day 20 (Rasmussen et al., 2011). Hu et al. (2010) reported that the anti-nociceptive effect of CTX (200 mg/kg) administered for seven days is sustained up to 21 days in animal models of thermal hyperalgesia; however, CTX fails to improve mechanical allodynia (Hu et al., 2010). In mice bladder distension models, the anti-nociceptive effect observed after seven day treatment of CTX (200 mg/kg) (Figure 3.1-3.3) is not sustained on day 30 (Figure 3.8). Differences between the somatic and viscera pain pathways may help explain the short-term effect of CTX in visceral pain models. For example, visceral organs have dual afferent innervation and send sensory information to different spinal cord segments and supraspinal regions. An option to improve the efficacy of CTX may be found in prolonged and localized treatment. Indeed, Hu et al. (2010) showed no significant reversal of mechanical allodynia for 21 days with a five day intrathecal treatment of CTX, in contrast, Ramos et al. (2010) showed that continuous intrathecal administration of CTX (227 nM) in the lumbar spinal cord for 20 days was effective in reversing tactile allodynia (Hu et al., 2010; Ramos et al., 2010). Taken together these studies suggest that systemic injections of CTX are effective short-term at mitigating somatic and visceral pain immediately after seven days. However, more studies are needed to determine the pharmacodynamics of CTX in the CNS.

Overall, work described in this chapter suggests that the spinal cord is a site of action responsible for the reduced visceromotor response to urinary bladder distension in
animals treated with 1-wk CTX. However, the possibility that enhanced glutamate uptake occurs in DRG that may be involved in mitigating somatic (Jasmine et al., 2010; Carozzi, et al., 2011) and visceral pain states, disburses further exploration. Also, these studies do not dismiss the possibility that the brainstem or higher centers of the brain are responsible for mitigation, due to the ample evidence that several sites in the brainstem (locus coeruleus) and cortex (anterior cingulate cortex) in the brain are involved in the process of visceral nociception (Hayashida et al., 2010; Wang et al., 2009). The second study showed that modest increase in trafficking of glutamate receptor subunits, such as AMPA receptor subunit GluR1, may contribute to the hyperalgesia in the CYP model of chronic bladder inflammation. Over-expression of GLT-1 attenuated the enhanced trafficking of the GluR1 subunit to the neuronal membrane by removing excess glutamate in the synaptic cleft (less activation of post-synaptic glutamate receptors) caused by CYP and may be a factor linked to abolished hyperalgesia after noxious/inflammatory stimulation to the bladder. The third study showed that 1-wk CTX in adult mice was effective at reducing neonatal stress-induced hyperalgesia to colonic distension. Finally, results suggest that the anti-nociception effect of 1-wk CTX is no longer present after 30 days, suggesting the need for continuous CTX administration for beneficial effects.
Chapter 4

Adeno-associated virus serotype 9 mediated transduction of EAAT2 gene attenuates visceralnociception

4.1 Introduction

The activation of first order pain-transmitting afferents leads to a release of the excitatory neurotransmitter glutamate at the first central synapse at the spinal cord (Yang et al., 1996). Hyperactivity of the glutamatergic neurotransmission contributes to the emergence of pathological visceral pain (Willert et al., 2004; Schicho et al., 2005). Previously, our laboratory and others demonstrated that transgenic and pharmacologic up-regulation of predominant glutamate transporter GLT-1 (human homologue EAAT2) was effective at mitigating colonic (Lin et al., 2009) and bladder (Yang and Roman, 2011) nociception in mice. Moreover, according to the data in section 3.1 the spinal cord and/or dorsal root ganglia are putative sites of action of GLT-1 over-expression to mitigate visceral nociception (Figure 3.1A-C). Advancements in viral-mediated gene therapy by adeno-associated viruses (AAV) have demonstrated improved targeting of virus delivery to astrocytes that may facilitate the cell-specific transduction of EAAT2. Therefore, AAV carrying the glutamate transporter EAAT2 gene may be a novel
approach to mitigate visceral pain and prolong the anti-nociceptive effects of EAAT2 over-expression.

Several viral vectors have been used as gene delivery vehicles for pain: adenovirus, lentivirus, herpes simplex virus (HSV), and adeno-associated virus. For example, adenovirus gene delivery of GLT-1 via intrathecal injection in rats showed diminished thermal hyperalgesia after carrageenan injections to the hindpaw (inflammatory) and mitigated the tactile allodynia produced after partial sciatic nerve ligation (Maeda et al., 2008). Also, lentivirus constructs that suppress nuclear factor-κ B (NF-κB) in the spinal cord of rats show a diminished response to models of pain after chronic constrictive injury (CCI) and a decreased expression of proinflammatory cytokines (Meunier et al., 2007). Moreover, the herpes simplex virus has been proposed for gene therapy of chronic pain due to its ability to incorporate large genomes (Manservigi et al., 2010). HSV studies in neuropathic pain models include successful gene delivery and over-expression of glial-derived neurotrophic factor (GDNF) (Hao et al., 2003), tumor necrosis factor-alpha (TNF-α) antagonist (Hao et al., 2007), enkephalin (Chattopadhyay et al., 2011) and glutamic acid decarboxylase (GAD) (Hao et al., 2005).

Some of these viral vectors possess properties that limit their potential use in clinical trials. Adenoviruses tend to elicit an innate and adaptive immune response that leads to undesired short-term gene expression (Kushwah et al., 2008, Muruve et al., 1999 and 2008). Similarly, studies of neuropathic pain reveal that HSV gene delivery is maintained only for a limited time (Hao et al., 2003). This is a common problem in viral therapy due to the degradation and ubiquinatation of their capsides, which results in limited
gene transfer (Heilbronn et al., 2010). Although lentivirus gene delivery can be long-term, issues remain with insertional mutagenesis (Heilbronn et al., 2010) and eliciting inflammation from transduced tissue (Seppen et al., 2006).

Numerous reports substantiate the efficacy of adeno-associated virus serotypes in transduction of cells, having a low immunogenicity (Xu et al., 2003), increased purification at high titers, and long-lasting transgene expression (Rivera et al., 2005). However, AAVs ability to cross the blood brain barrier (BBB) differs across serotypes (Manfredsson et al., 2009). Recently, studies showed that the AAV9 serotype can cross the BBB in mice (Foust et al., 2009), pigs, and macaque monkeys (Bevan et al., 2011) after systemic administration. Moreover, the AAV9 serotype can preferentially target astrocytic cells in the CNS of adult mice (Foust et al., 2009). In addition, AAV9 transduction of neuronal and glial cells is conserved across species. Therefore, the AAV9 serotype is a promising option for targeting astrocytes for gene delivery of GLT-1 in the study of anti-nociception.

Studies investigating the effectiveness of gene therapy as therapeutic treatment for visceral pain are lacking. Although previous studies have used adenoviruses for gene delivery of GLT-1 in the CNS in neuropathic pain models (Maeda et al., 2008), research on the potential of AAV as therapeutic option for visceral pain is in its infancy. Since AAV serotype 9 is an improved viral vector for gene delivery to astrocytic cells and has the capacity for long-term gene delivery of the glutamate transporter EAAT2 (human GLT-1 homologue) to the CNS, the effect on visceral nociception needs to be explored. Given the success of enhanced glutamate transporter GLT-1 activity to mitigate visceral
nociception (see section 3.2), this chapter will address the effects of augmentation of EAAT2 expression via gene therapy, thereby enhancing glutamate uptake as a strategy to mitigate visceral nociception. Furthermore, these important preliminary studies will help build toward localized injection of the AAV9-EAAT2 in the spinal cord or DRG.

4.2 Methods

4.2.1 Animals. All studies used female CR57/BL mice (20g-25g) supplied by Harlan (Indianapolis, IN) and were housed together (littermates) in cages with bedding and free access to food and water. Animals were monitored twice daily (10 a.m. and 5 p.m.) for changes in appearance (e.g., infections, fur loss, self-inflicted injuries, weight loss). Pups were weaned at 21 days. Guidelines for animal care were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and followed the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

4.2.2 AAV9 production. Double stranded AAV2-ITR based CB-GFP or EAAT2 vector was used to produce the self-complementary AAV9, with a plasmid encoding Rep9cap9 sequence and an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells; moreover, sequencing was used to verify the AAV serotype as previously described (Gao et al., 2004). Separate batches of the virus were produced by a manufacturing company (Virapur LLC, San Diego, CA) that purified, dialyzed against PBS, and formulated the virus to prevent aggregation. Vector preparations were titered by Taq-Man
technology (quantitative–PCR) and the purity of the vector was determined by silver staining (Invitrogen, CA).

4.2.3 Intravascular vector delivery. The dams (single housed) were separated from their litter on postnatal day 15-20. Mice were placed in a restraint device that allowed the tail to receive focused lighting for easier localization of the tail vein. Prior to receiving an injection the tail was swabbed with an isopropynal alcohol gauze and a 30G insulin syringe containing either 100 µL of PBS, AAV9-GFP, or AAV9-EAAT2 was inserted into the tail vein and delivered 4x10^{11} vg/kg of viral solution. The animals were then returned to separate cages and observed for discoloration of the tail.

4.2.4 CRD surgery. Surgery was performed as previously described in chapter 2. Briefly, animals were anesthetized by intraperitoneal injections of Ketamine (137 mg/kg; Hospira) and Xylazine (20 mg/kg; Bayer). An EMG electrode wire (Cooner Wire, CA) was stripped from both ends and inserted into the external oblique muscle. One end of the wire was securely tied to the muscle with suture thread (6-0 silk) and the same procedure was repeated with a second wire that was placed (0.5 cm) next to the first wire. The two wires were then tunneled underneath the skin and emerged from the neck area. The electrode wires were secured to the superficial muscle of the neck by a knot of suture. The incisions in the abdomen and neck were closed by surgical clips (Michael Clips). Animals were allowed to recover for a minimum of three days from surgery and were housed alone to avoid tampering of the wires.

4.2.5 CRD experiment. Colorectal distension was performed as previously described in chapter 3. Briefly, animals were anesthetized with 4% isoflurane (adjusted to 1.5% for
most of the procedure). A lubricated sheath (PE-240 tube; 4 cm long) snugly covering a balloon attached to a PE-60 tube (10 cm) was inserted into the distal colon (2.5 cm) and the protective sheath was removed. The balloon was secured in place by taping the exposed PE-60 tube to the base of the tail. The EMG electrodes protruding from the neck and protected with a Teflon coating were stripped to expose wire filaments. Animals were placed inside restraint devices, previously described in section 2.2.5. The electrodes were attached to a recording apparatus (see EMG recordings for details) and animals were allowed to recover and acclimate for 1 hr before commencing CRD. The visceromotor response to colorectal distension was elicited by intracolonic balloon distension at 15, 30, 45, and 60 mm Hg (10 sec baseline recording; 40 sec actual distension), each distension pressure was repeated three times with 3 minute intervals. Data was analyzed as the number of EMG spikes above baseline with Spike2 software (Cambridge Electronic Design, Cambridge, UK).

4.2.6 Western blot. Animals were euthanized according to IACUC guidelines and the lumbosacral region (L5-S2) of the spinal cord was removed for processing. The spinal cord was homogenized in 1X lysis buffer (cell signaling technology) containing 1X complete protease inhibitor cocktail (Roche Lot.11714800). Protein concentration was determined via Bradford assay and 70 µg of sample was loaded in 8% SDS polyacrylamide gel and wet transferred to nitrocellulose paper overnight at 4°C. The membrane was placed in 3% Milk-TBST blocking buffer for 1 hour. The membrane was exposed to rabbit anti-EAAT2 antibody (1:1,000; Santa Cruz) primary antibody in 3% milk-TBST overnight at 4°C. The membrane was washed for 15 minutes 3 times with
TBST. Afterwards, the membrane was exposed for 1.5 hr to horseradish peroxidase (HRP)-conjugated goat IgG (1:3,000; Bio-Rad) secondary antibody. Enhanced Chemiluminescent Substrate (GE Healthcare Amersham; RPN2106) was used for HRP signal detection. The membrane was then stripped with acidic buffers (1 hr) to remove primary and secondary antibodies, rinsed, blocked (3% non fat milk) and probed with rabbit anti-actin antibody (1:3,000; Santa Cruz) primary antibody overnight at 4°C, followed with secondary antibody probing (mentioned before), 3X wash and ECL exposure. Densitometry was performed with Bio-Rad Quantity One 1-D analysis software.

4.2.7 Tissue processing. Spinal cord extraction occurred between 30-35 days post injection. Animals were anesthetized with pentobarbital solution and reflex responses were checked prior to performing midventral sternal thoracotomy to expose the heart. A cannula (PE-20 tube) was placed in the aorta via the left ventricle and secured into place with a clamp. A small incision was made on the atrium and 20 mL of PBS was allowed to flow at a constant rate through the cannula. PBS perfusion was stopped until the liver of the animal was pink in appearance. The animal was then perfused (50 mL) with 4% paraformaldehyde in PBS (pH=7.4). Tissue was post-fixed in 4% paraformaldehyde for 24 hours prior to sectioning.

4.2.8 Immunofluorescent microscopy. The lumbosacral regions of the spinal cord were embedded in 3% agarose and sectioned into 25 µm transverse sections with a Leica VT1200 vibrating microtome. Sections were placed in 96 well plates containing Tris-buffered saline. The sections were then transferred into a blocking solution (10% donkey
serum, 1% triton-X100 in tris-buffer saline) for 1 hour. Afterwards, sections were incubated with either rabbit-anti-GFP (1:500; Invitrogen) or guinea-pig-anti-GFAP (1:1,000; Advanced Immunohistochemical) primary antibodies overnight at 4°C. Primary antibodies were detected via Fic- or Cy3-conjugated antibodies (1:1,000; Jackson ImmunoResearch). A Zeiss 710 Meta confocal microscope (Car Zeiss MicroImaging, NY) was used to generate fluorescent images and processed with LSM software.

4.2.9 Glutamate uptake assay. Animals were euthanized 35 days after tail vein injections. The lumbosacral region of the spinal cord was removed and placed in a tissue homogenizer containing 3 mL tissue buffer at 4°C (0.32 M sucrose, 0.05 M Tris-base pH 7.4 and proteinase inhibitor cocktail). The tissue was homogenized via 15 strokes of a pestle attached to a homogenizer motor. The homogenized tissue was placed in to two 1.5 mL eppendorf tubes (Fisher-Scientific) and centrifuged at 1,000 g for 10 min at 4°C. The bottom pellet was discarded and the supernatant containing synaptosomes was collected and centrifuged at 16,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in either 1 mL of Kreb buffer (120 mM of NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 25 mM NaHCO₃, 0.55 D-glucose, 2 mM CaCl₂ at pH=4) or 1 mL of Na⁺ free Kreb buffer (120 mM choline-Cl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 25 mM Tris-Cl, 0.55 mM D-Glucose, 2 mM CaCl₂ at pH=7.4). The synaptosomes were pre-incubated at 37°C for 10 minutes and 0.1 mM glutamate and 0.05 µM tritium-labeled glutamate 1 mCi/mL (Perkinelmer Inc.) was added into each tube and incubated for 10 min at 37°C. To halt the uptake of “hot” glutamate 500 µL of tissue buffer at 4°C was added to each tube and then placed on ice bucket. Synaptosome
suspension was then transferred to GF/B (Watman, UK, no.1821024) glass microfiber filter paper pre-coated with 0.2% polyethylene solution via vacuum filtration. The filter paper was washed three times with 4 mL of PBS buffer at 4°C and placed inside scintillation vials containing 3 mL of scintillation buffer. A scintillation counter (Beckmann, USA) was used to determine the decay of the tritium labeled glutamate found in synaptosomes. To calculate total GLT-1 glutamate uptake the sodium dependent glutamate uptake (control) was subtracted by the glutamate uptake of the Na⁺-free group.

4.2.10 Statistics. The student t-test (two-tailed unpaired) was used to determine significant differences in the glutamate uptake assay. A random block design with a one-way ANOVA was used for behavioral experiments. Analysis of variance was followed by Least Significant Difference (LSD) comparison post-hoc test. Bio-Rad Quantity One software was used to determine optical density of western blots. Moreover, the optical densities of protein blots were analyzed by standardizing the optical density of PBS or AAV9 against the optical density of the β-actin protein. The quantitative data in each study was expressed as mean ± SEM; data were considered statistically significant different if p<0.05.

4.3 Results

4.3.1 Neonatal intravenous AAV9 targets multiple cell types in the spinal cord

Tail vein injection of AAV9-GFP 4X10¹¹ in mice (P15-20) successfully crossed the blood-brain-barrier, evident by the overall transgene expression of the parenchyma
and GFP labeling. Sparse co-localization of GFAP and AAV9-GFP (yellow) was observed in the superficial dorsal horn and dorsal column of the spinal cord (Figure 4.1). There is also minor transduction of astrocytic cells (yellow) in the superficial dorsal horn 35 days after injection (Figure 4.1 inset).

**Figure 4.1. AAV9-GFP mediates transgene expression in the spinal cord.** Intravenous injection of AAV9-GFP (green) showed GFP expression in the lumbosacral region after 35 days (bar represents 100 µm). The inset picture shows GFP (green), labeled astrocyte (red), and colabeling (yellow). AAV9-GFP tail vein injection in mice showed transduction into astrocytic cells (inset) and overall spread in the spinal cord.
4.3.2 Increased expression and glutamate uptake of EAAT2 after AAV9-EAAT2 transduction

Since the spinal cord is a putative site of action of GLT-1 upregulation to mitigate visceral nociception (see section 3.1), the spinal cord was assessed in these experiments. Hence, to determine whether AAV9-EAAT2 increased EAAT2 protein expression in the lumbosacral region of the spinal cord compared to mice that received PBS or AAV9-GFP tail vein injections an immunoblot was performed (Figure 4.2). The data revealed that AAV9-EAAT2 (n=2) treated mice have an increased expression (17%) compared to PBS (n=2) or AAV9-GFP (n=1). Therefore, gene delivery of EAAT2 by AAV9-EAAT2 leads to increased expression of EAAT2 in the spinal cord; 35 days after tail vein injection.

![Figure 4.2. AAV9-EAAT2 induced glutamate transporter over-expression.](image)

Representative western blot comparing EAAT2 protein expression in lumbosacral region of mice that received tail vein injection (100 µL) of either PBS, AAV9-GFP, or AAV9-EAAT2.

To investigate whether glutamate uptake activity was affected by AAV9-EAAT2 gene delivery a synaptosomal glutamate reuptake assay was performed in the spinal cord. The data shows that on day 35 post-injection, animals that received AAV9-EAAT2
treatment had a 105% increase (*p<0.05) in EAAT2 mediated glutamate uptake compared to control (Figure 4.3).

Figure 4.3. AAV9-EAAT2 enhanced glutamate uptake. Increased glutamate sodium mediated uptake was observed in mice that receive tail vein injections (100 µL) of AAV9-EAAT2. *p<0.05

4.3.3 EAAT2 gene delivery mitigated the visceromotor response to CRD

Evoked visceromotor responses to graded colo-rectal distension from 15 to 60 mm Hg were elicited in animals 35 days after either PBS or AAV9-EAAT2 intravenous injection. Neonatal intravenous injection of AAV9-EAAT2 significantly reduced the evoked VMR to CRD in adult mice at 60 mm Hg compared to PBS control (71% change) (Figure 4.4 *p<0.05). There was an insignificant trend towards a reduced nociceptive
response to colo-rectal distension in animals treated with AAV9-EAAT2 compared to intravenous PBS cohorts at 15 and 45 mm Hg distension pressures. Animal cohorts treated with AAV9-GFP produced responses not different from PBS control.

Figure 4.4. AAV9-EAAT2 decreased visceromotor response to CRD. The visceromotor response to colo-rectal distension shows a clear graded response to increasing CRD pressures in control (PBS) mice 35 days after treatment (100 µL). AAV9-EAAT2 (4X10^{11} vg/kg) reduces (71%) visceromotor response at 60 mm Hg compared to PBS control (*p<0.05).
4.4 Discussion

Systemic administration of ceftriaxone (CTX), a substance that over-expresses GLT-1, mitigates both somatic (Hu et al., 2010; Ramos et al., 2010) and visceral (Lin et al., 2009; Lin et al., 2011; Yang and Roman, 2011) nociception in rodents. Viral-mediated transduction of EAAT2 (GLT-1) represents an important translationally relevant advance to pain therapeutics. The emergence and advances in viral vector development has illuminated the potential of gene delivery to desired cells and tissues to produce sustained protein expression. Previous studies demonstrated that most serotypes of AAV can be used for long-term gene delivery and, unlike other viral vectors, AAV produces little immune response (Moscioni et al., 2006; McCaffrey et al., 2008). Although previous studies have demonstrated successful transgene expression of AAV9-GFP 10 or 21 days after after systemic injection (Foust et al., 2009), gene delivery and long-term expression can vary due to normal physiological development in mice. In this chapter the data demonstrated that intravenous administration of AAV9-GFP into neonatal (P15-20) mice can successfully transduce multiple cells of the lumbosacral segment of the spinal cord (Figure 4.1). The majority of sparse co-labeling with astrocytic marker GFAP was observed in the superficial dorsal horn. Although our findings differ from those reported by Foust et al. (2009), this might be attributed to differences in the postnatal day of administration of AAV9-GFP; P1 (Foust et al. 2009) vs. P15-20 (Chapter 4). The transgene expression pattern of AAV9-GFP in adult (~70 day old) mice is mostly astrocytic, while systemic injection of AAV9-GFP in neonates
(on postnatal day 1) tends to be neuronal and astrocytic (Foust et al., 2009). However, the effective targeting of AAV9 in adult mice is age-dependent and further studies are required to determine the optimal age of AAV9 transduction to relevant sites involved in visceral pain in the spinal cord, DRG, and supraspinal regions. Foust et al. (2010) showed that successful delivery of survival motor neuron (SMN) protein via scAAV9 was dependent on which postnatal day intravenous injections occurred (Foust et al., 2010).

Moreover, the site of injection is important in targeting cells in the CNS: intracranial injections target neuronal cells and tail vein injections target astrocytic and neuronal cells in mice (Foust et al., 2009). Despite the differences, results show that delivery of AAV9-GFP up to postnatal day 20 is effective at crossing the blood brain barrier and transduce the superficial dorsal horn, an area relevant for visceral pain transmission.

Given the small gain seen in attenuating the VMR to 60 mm Hg colonic distension an improved delivery method via intrathecal administration may be a viable option for future work. Spinal cord or DRG was characterized as a site of action of GLT-1 over-expression to mitigate visceral nociception (Lin et al., 2011; section 3.1). Recent reports suggest that spinal AAV9 transduction occurs after intrathecal injections in 5 day or 2.5 month old pigs and that gene delivery is dramatically improved (Bevin et al., 2011; Fedirici et al., 2011). Although the preliminary studies found in this chapter cannot establish if AAV9 can target the same region of the spinal cord in much older mice (>8 weeks) or via different delivery routes, AAV9 is an ideal candidate for investigating new therapeutic options in treating visceral pain due to its ability to cross the blood-brain-
barrier in adult mice 70 days old and 3-year old macaque monkeys (Foust et al., 2009; Bevan et al., 2011; Gray et al. 2011).

A common occurrence in visceral and somatic pain states is an increase in spinal nociceptive hyperexcitibility that involves the overstimulation of glutamatergic receptors (NMDA, AMPA, mGLURs). Therefore, changes in synaptic glutamate concentration due to excess glutamate release from glutamatergic nerve endings are efficiently restored by the glutamate transporter GLT-1 (EAAT2), predominantly located in astrocytic cells (Lehre et al., 1998). Moreover, GLT-1 (EAAT2) is responsible for more than 90% of total glutamate uptake in the CNS (Bar-Peled et al., 1997; Maragakis et al., 2005). Hence, controlling glutamate levels in the synaptic cleft by facilitating glutamate uptake may help decrease visceral pain. Indeed, our data suggests administration of AAV9-EAAT2 treatment lead to an increase in EAAT2 expression (Figure 4.2) and glutamate uptake (Figure 4.3) in the lumbosacral region of the spinal cord that corresponds to distal colon afferent innervation via the pelvic and hypogastric nerves (see Figure 1.2 in chapter 1).

Visceromotor responses generated by graded CRD pressures and quantified by EMG recordings are reduced at 60 mm Hg in mice 35 days after receiving AAV9-EAAT2 compared to the PBS control group (Figure 4.4), which is consistent with reports that EAAT2 (mouse homologue GLT-1) is important for attenuating visceral nociception (Lin et al., 2009; Yang and Roman, 2011). This study also reports similar findings to those in models of somatic pain by adenoviral delivery of GLT-1 in rodents (Maeda et al., 2008). In addition, the data suggests that viral vector gene delivery of (human GLT-1 homologue) EAAT2 can offer long-term protection against noxious stimuli to the colon.
with slight modifications to the protocol. Although we did not observe a significant
difference in VMR at 15-45 mmHg in animals treated with AAV9-EAAT2, this may be
related to 1) insufficient administered virus titer to sustain the time required for long-term
effect of AAV9-EAAT2 transduction need to be optimized in future studies. Of note,
over-expression of GLT-1 (EAAT2) at the current titer concentration may be enough to
attenuate models of visceral hyperalgesia without affecting the normal VMR response to
noxious colo-rectal distension; a topic of future research. Perhaps a higher volume titer
of the AAV9-EAAT2 may yield improved results to lower distension pressures. Indeed,
previous research showed that spread of transduction is closely linked to injections site,
volume, and titer variations (Federici et al., 2011). Hence, localized administration of
AAV9-EAAT2 may increase transgene expression and reduce virus instability and
degradation.

This study did not investigate nociception of other visceral organs beside the
colon, the distal colon shares its afferents (mainly pelvic nerve) with the bladder and their
 terminals are found in close proximity in the spinal cord (Christianson et al., 2010;
Miranda et al., 2011) and a study on the effect of AAV9-EAAT2 transduction on bladder
distension may find similar results to that of the CRD model. Furthermore, we cannot
dismiss the possibility that AAV9-EAAT2 gene delivery to cells via the brain or
brainstem may be an important delivery route for improving and advancing therapeutic
outcomes by viral vectors. However, future studies exploring gene delivery are
encouraged by these preliminary results to improve region specific targeting of viral vectors (e.g., neurons specific to the VPL).

In conclusion, neonatal intravenous AAV9-EAAT2 reduces the VMR to CRD presumably due to increased expression and activity of predominant glutamate transporter GLT-1 (EAAT2). Overall the use of gene therapy to treat chronic visceral pain is in its infancy and the studies presented in this dissertation are the first to use scAAV9 as a model for EAAT2 gene delivery to spinal cells and attenuation of visceral pain. These studies suggests, that the over-expression of predominant glutamate transporter GLT-1 (EAAT2) and subsequent increased glutamate uptake in the spinal extracellular space can be sustained 35 days after administration and as a consequence mitigate the visceromotor response after noxious colo-rectal distension.
Chapter 5

Conclusions and Perspectives

5.1 Perspective

Glutamate is the principal neurotransmitter mediating somatic and visceral sensory signaling; aberration of glutamatergic systems can generate modifications of spinal neuronal activity that contribute to analgesia (PAG), hyperalgesia, and allodynia (Behbehani and Fields, 1979; Minami et al., 1994; Weng et al., 2005). Glutamate transporters maintain glutamate homeostasis in the CNS and are important for preventing glutamate spillover to adjacent synapses (Sullivan et al., 2004). Studies on neuropathic pain have shown that GLT-1 over-expression mitigates hyperalgesia and allodynia (Hu et al., 2009; Ramos et al., 2010; Maeda et al., 2008). The potential role of enhanced glutamate transporter activity to mitigate visceral nociception has been advanced by work conducted in this dissertation and the work of others (Lin et al., 2009; Lin et al., 2011; Yang and Roman, 2011). Therefore, this dissertation establishes the importance of GLT-1 over-expression in mitigating visceral nociception in animal models of acute and chronic visceral pain.
Management of persistant pain typically involves the use of opioid drugs with side effects that include addiction, development of tolerance, constipation, somnolence, and death due to respiratory failure (Ballantyne and Mao, 2003). Thus, there is great interest in developing new compounds that can mitigate pain with minimal side effects. The compound ceftriaxone enhances the expression of the glutamate transporter GLT-1 throughout the CNS and can reverse opioid induced hyperalgesia in mice (Chen et al., 2011). Due to the importance of GLT-1 in modulating glutamate concentration at the synaptic level, this dissertation explored pharmacological and gene therapy approaches to induce anti-nociceptive effects via GLT-1 over-expression.

To date, researchers are dependent on animal models of visceral pain for studying mechanism and screening potential anti-nociceptive compounds. Indeed, this dissertation studied animal models of visceral pain that are based on mechanical (distension) or chemical noxious stimulation of visceral organs. However, these animal models have limitations since they are based on techniques that require either surgical intervention, in the case of EMG recordings, or subjective behavioral monitoring during chemical irritation. Recently, Larauche et al. (2010) reported an alternative non-invasive procedure to record changes in visceral sensitivity in conscious mice by recording the intraluminal colonic pressure (ICP) using a pressure sensor. Although Larauche et al. (2010) reported some differences between invasive vs. non-invasive procedures, the experimental conditions conducted in this dissertation were identical for the control and experimental cohorts (housing, food, surgical procedures, etc). Thus, responses are not confounded by the surgical procedure utilized. Moreover, this dissertation showed that despite the
modest increase in VMR due to UBD or CRD that may occur in the control groups due to surgical procedures, this increase is not significant enough to mask the obvious difference in VMR recorded in animal groups that received visceral noxious stimuli and the data demonstrating GLT-1 over-expression mitigates enhanced visceral sensitivity.

The studies conducted in this dissertation used either FVB/N (chapters 2 and 3) or CR57/BL (chapter 4) mice. FVB/N mice are susceptible to colon inflammation via DSS (Neufert et al., 2007). However, CR57/BL/6 are resistant to TNBS induced colitis (Scheiffele and Fuss, 2002). Therefore, FVB/N mice are preferred for experiments that involve inflammation of visceral organs. The majority of the literature regarding transduction of AAV9 is based on CR57/BL mice (Foust et al., 2009; Duque et al., 2009; Miyake et al., 2011). Several factors determine the spread/efficacy of the AAV9 in the CNS: delivery route, titer concentration, age of mouse, etc (Pacak et al., 2008; Foust et al., 2009; Snyder 2011). Hence, to avoid issues with strain differences the CR57/BL mice were used in chapter 4.

Although non-invasive procedures are preferred when developing animal models that study pain, all current animal models have short comings and are widely debated topics (Mogil, 2009). Nevertheless, there is emerging evidence that suggest similarities of brainstem activation in human and animal models of visceral and somatic pain, such as fMRI studies conducted in humans showing comparable regions activated after colonic distension (Dunckley et al., 2005). In addition, there are similarities in the activation of the prefrontal cortex between humans and animal visceral pain models of colonic distension, mainly cell activity is enhanced in regions of the intralimbic, prelimbic, and
rostral anterior cingulate cortex (Silverman et al., 1997; Baciu et al., 1999; Gibney et al., 2010). In regional cerebral blood-flow mapping studies, Wang et al. (2009) reported several similarities between rat and human brain activation patterns to noxious visceral stimuli (Wang et al., 2009).

Although this dissertation relied on bladder exposure to acrolein as a model of interstitial cystitis, it is important to establish that most patients with interstitial cystitis do not have bladder inflammation. However, patients that receive cyclophosphamide treatment for tumor suppression develop bladder inflammation that leads to interstitial cystitis. Therefore, research on cyclophosphamide induced interstitial cystitis may be limited to a small percentage with pathophysiological conditions. Nevertheless, this dissertation establishes the anti-nociceptive effects of GLT-1 over-expression and future studies should include alternative animal models of visceral pain (i.e., chronic pancreatitis and cholecystitis) that may benefit from our exciting findings.

5.2 Conclusions and future directions

This dissertation demonstrated the importance of GLT-1 over-expression in modulating visceral hyperalgesia via pharmacological and gene therapy techniques. First, this dissertation confirmed a clear GLT-1 over-expression and increased glutamate uptake activity in animals treated with a pharmacological compound (Figure 1.1 A-B) and gene therapy (Figure 4.2; Figure 4.3). The pharmacological data parallels the results generated by Rothstein et al. (2005) describing the enhanced expression and activity of
GLT-1 by 1-wk CTX treatment (Rothstein et al., 2005). Previous studies in transgenic and pharmacological mice models of GLT-1 over-expression showed an attenuated visceromotor response to colo-rectal distension (Lin et al., 2009; Lin et al., 2011). A similar effect was reported in this dissertation, after 1-wk CTX (200 mg/kg) treatment the visceromotor response to urinary bladder distension is reduced compared to control cohorts (Figure 1.2). Also, studies demonstrate that increased visceral hypersensitivity via TNBS induced inflammation is reduced after 1-wk CTX treatment (Lin et al., 2011). Similarly, acrolein (0.4 mM) induced bladder irritation (Figure 2.4), or inflammation (Figure 2.5) increased the visceromotor response to graded bladder distension (p<0.05) and was attenuated by 1-wk CTX treatment at a dose of 200 mg/kg compared to control cohorts.

Although the expression and uptake activity of GLT-1 after systemic injections of 1-wk CTX at doses 100 mg/kg or 50 mg/kg was not reported in this dissertation, there is evidence that systemic injections of CTX for five days at a dose of 100 mg/kg increased GLT-1 uptake and expression in the nucleus accumbens (Rasmussen et al., 2011). Data collected from animals that received a lower dose (100 mg/kg) of 1-wk CTX treatment in models of bladder inflammation (Figure 2.6) or persistent inflammation (Figure 2.7) showed a diminished visceromotor response to urinary bladder distension. Therefore, future studies should quantitate glutamate uptake activity of GLT-1 in the lumbosacral spinal cord region after 1-wk CTX at 100 mg/kg and 50 mg/kg.

In addition, the time spent licking of the abdominal area after acrolein (0.4 mM) infusion to the bladder was enhanced compare to control cohorts and may be indicative of
supraspinal and spinal circuitry modulation (Figure 2.6). However, 1-wk CTX treatment at a low dose (100 mg/kg) diminished the time spent licking. Although inflammation of the bladder was not determined by histological methods in these studies, several reports indicate that lymphocyte infiltration and ulceration of the bladder epithelial wall occurs after repeated systemic administration of cyclophosphamide due to the toxic metabolite acrolein accumulating inside the bladder of mice (Wang et al., 2008; Lai et al., 2011; Boudes et al., 2011). Since GLT-1 over-expression via 1-wk CTX treatment is effective at reducing visceral nociception in animal models of inflammatory bowel disease (TNBS) and interstitial cystitis (cyclophosphamide), there is a possibility of observing similar affects in other models with relevance to the pathophysiology of persistent visceral pain. Behavioral animal models of adult posttraumatic stress show that the development of chronic visceral pain is common and can be monitored by EMG (Bradesi et al., 2005) or intraluminal colonic pressure recordings (Larauche et al., 2010). Thus, GLT-1 over-expression could be studied for efficacy in enhanced nociception associated with posttraumatic stress.

The complex organization of the visceral afferents terminals in the spinal cord is evident in patients that experience referred pain due to the manifestation of cross-organ sensitization (see section 1.5). Although several theories have been postulated (Figure 1.4-1.5) to explain the occurrence of cross-organ sensitization, there is no evidence that glutamate modulation may reduce cross-organ sensitization. Therefore, this dissertation determined that GLT-1 over-expression and increased glutamate uptake activity (Figure
2.1) via 1-wk CTX treatment attenuates cross-organ sensitization as a result of colon inflammation via TNBS (Figure 2.9A-B).

Although the visceral pain pathway is largely dependent on glutamate to regulate visceral nociception, the site in the central or peripheral nervous system (e.g., dorsal root ganglia) that is responsible for reducing visceral pain via GLT-1 over-expression is unclear. However, studies have demonstrated that systemic and intrathecal injection of DHK (a selective GLT-1 antagonist) reverses the effect of 1-wk CTX (200 mg/kg) treatment in colo-rectal distension models (Lin et al., 2009; Lin et al., 2011). The reversal of 1-wk CTX treatment after intrathecal DHK suggests a spinal cord or peripheral sensory mechanism may be responsible for the attenuated visceromotor response to noxious colo-rectal distension. The bladder, a visceral organ that shares sensory pathways with the distal colon (see section 1.5), can expand on the concept of a spinal or peripheral site of action by demonstrating a similar reversal of the 1-wk CTX anti-nociceptive effect after intrathecal DHK treatment. Indeed, this dissertation verified that the region responsible for the anti-nociceptive effect may reside in the lumbosacral segment of the spinal cord or DRGs by reversing the anti-nociceptive response caused by GLT-1 over-expression via intrathecal (Figure 3.1-3.3) or systemic (Figure 2.3) injections of dihyrokinic acid.

An important mechanism to establish hypersensitivity from either somatic or visceral tissue is central sensitization. Central sensitization is characterized by increased excibility (action potentials) of dorsal horn neurons that involves activation of glutamate receptors (Mayer et al., 1985; Mayer et al., 1987; Zuromboski et al., 1989) found in the
postsynaptic membrane. A major mechanism in establishing central sensitization is increased trafficking of receptors to the membrane. Although NMDA glutamate receptor and its subunits NR1 and NR2A play a vital role in central sensitization, the AMPA glutamate receptor and subunit GluR1 may contribute to initiation and maintenance by increasing calcium permeability and reducing the threshold activation of dorsal horn neurons in persistent inflammation models of pain (Kopach et al., 2011). Galan et al. (2004) showed an increased GluR1 expression in the membrane up to 180 minutes after intracolonic administration of capsaicin; an acute model of visceral pain (Galan et al., 2004). Although the role of GluR1 trafficking in chronic visceral pain is poorly understood, the data suggests that GluR1 trafficking to the membrane is increased after prolonged inflammation of the bladder via repeated treatments of cyclophosphamide (Figure 3.4-3.5). However, the results cannot determine whether the increased GluR1 subunit expression occurred in the postsynaptic or extrasynaptic membrane; or both. In models of persistent somatic inflammation the increased GluR1 expression is associated with changes in the extrasynaptic membrane (Kopach et al., 2011). Also, it is known that AMPA GluR1 homomeric receptors are more abundant in the extrasynaptic membrane than in the postsynaptic membrane (Arendt et al., 2010; Kopach et al., 2011). However, a possible outcome of persistent inflammation states is the insertion of calcium permeable AMPA receptors (GluR1 homomeric) from the extrasynaptic pool to the postsynaptic membrane that contribute to synaptic plasticity (Kopach et al., 2011). Also, this dissertation established that tight modulation of glutamate by glutamate transporters limits the trafficking or insertion of GluR1 to the membrane after persistent
inflammation of the bladder, since elevated trafficking of GluR1 to the membrane was diminished by 1-wk CTX treatment (Figures 3.4 and 3.5). Under neuropathological conditions excess glutamate release by primary efferents may spillover to extrasynaptic sites and increase insertion of the calcium permeable AMPA receptors to the postsynaptic membrane. Hence, enhanced expression of the GluR1 subunit in prolonged visceral pain states may implicate a maintenance role in central sensitization. However, intracellular posttranslational modifications (e.g., phosphorylation of subunits) of AMPA subunits may also be involved in enhanced activity of the AMPA receptor in conditions of hyperalgesia and future aims should determine whether GLT-1 over-expression modulates these modifications.

Several studies have reported that early-life stress due to chemical noxious stimulation of a visceral organ or maternal separation facilitates the induction of visceral hypersensitivity and changes colon function in adult rodents (Al-Chaer et al., 2000; Gosselin et al., 2010; Christianson et al., 2010; Moloney et al., 2012). However, the role of glutamate in these models of prolonged susceptibility to visceral hypersensitivity has not been explored. Animals administered 2% mustard oil on postnatal day 9 and 11 show a 5-7 fold increased in visceromotor response to colo-rectal distension (30-60 mmHg) as adult mice (8 weeks old) compared to control cohorts. However, mice that received 2% mustard oil treatment on P9 and P11 but were treated with CTX (200 mg/kg) seven days prior to undergoing colo-rectal distension had a diminished visceromotor response, indicating an attenuation of visceral hypersensitivity via a glutamate-mediated mechanism. Although this experiment did not include histological evaluations of the
colon after 2% mustard oil treatment, previous studies measuring MPO activity have noted that 2% mustard oil does not induce inflammation (Christianson et al., 2010). Instead, mustard oil elicits irritation of the colon via activation of TRPA1 receptors (Christianson et al., 2010). The main mechanisms that are involved in increasing visceral hypersensitivity in irritable bowel syndrome due to early life stress are not clear. However, it is accepted that plasticity in the hypothalamic-pituitary-adrenal axis and cortex are involved. Clinical studies show that patients with irritable bowel syndrome have a diminished grey area density in the medial and ventrolateral prefrontal cortex (Seminowicz et al., 2010). These cortical areas are important for descending inhibition sensory pathways and poor synaptic modulation may play a role in susceptibility to visceral hypersensitivity. Although the early-life stress model used in this dissertation showed a clear enhancement of visceral hypersensitivity in adult mice due to exposure to a chemical irritant as neonates (Figure 3.7), the effect of 1-wk CTX on adult mice that experienced maternal separation should be explored in future studies.

Previous studies conducted in neuropathic pain models reported that the CTX effect lasted up to 20 days and reduced thermal hyperalgesia (Hu et al., 2010). The duration of the anti-nociceptive effect of 1-wk CTX treatment (200 mg/kg) during graded urinary bladder distension was not sustained after 30 days in mice (Figure 3.8). However, the diminished effect of 1-wk CTX treatment after 30 days was not verified by measuring GLT-1 expression or uptake activity. A study in the nucleus accumbens of rats determined that five day CTX treatment enhanced glutamate removal up to day 20 (Rasmussen et al., 2011).
This dissertation piloted the novel use of adeno-associated virus serotype 9 to induce over-expression of EAAT2 (human GLT-1 homologue) (Figure 4.2) and increase spinal glutamate uptake (Figure 4.3). This dissertation also showed that AAV9 gene delivery of EAAT2 produced anti-nociceptive effects on (at 60 mm Hg) colo-rectal distension (Figure 4.4). Previous studies show that the optimal window of gene delivery can be age, titer, route, and promoter dependent (Pacak et al., 2008; Foust et al., 2010). Future studies will address enhanced targeting of astrocytes, age differences (neonatal vs adult), delivery methods (intrathecal vs. intravenous), long-term effects, and optimal volume of titer delivered to optimize the window of transduction.

5.3 Final remarks

Chronic visceral pain states are difficult to alleviate and patients seeking treatment often encounter few options, which can have severe side effects. The latest of these drugs, tegaserod maleate (Zelnorm), a short-term treatment for IBS in women, was removed from the market due to side effects. The explanation for this lack of options is in part due to few studies addressing the mechanisms of visceral pain, instead the bulk of studies are conducted on somatic pain. Although visceral and somatic pain share similar pathways and mechanisms, emerging studies reveal that differences are common.

Nevertheless, more experimental studies on visceral pain are required to tease out these differences to develop better therapeutic options. This dissertation attempts to highlight some of the challenging mechanisms of visceral pain and provide a therapeutic approach.
for treating acute and chronic visceral pain. Indeed, studies are emerging that confirm the
efficacy of GLT-1 over-expression in attenuating chronic visceral and somatic pain in
animal models (see section 1.10).

There is growing interest in the therapeutic potential of GLT-1 over-expression to
treat other neurodegenerative disorders. However, the clinical utility to modulate
 glutamate transporter activity depends on diminished side effects compare to current
opioid drugs. Studies on animal behavioral models show that GLT-1 over-expression
does not alter motor or cognitive function (Miller et al., 2008; Lin et al., 2011). Clinical
trials specific for treatment of visceral pain syndromes via GLT-1 over-expression must
be considered in the future.

Overall, the initial goals of this dissertation were realized by demonstrating the
widespread applications and benefits of pharmacological or gene therapy approaches to
treat visceral pain disorders by over-expressing the glutamate transporter GLT-1.
However, the potential analgesic utility of GLT-1 over-expression will require a greater
understanding of 1) the duration of anti-nociceptive effect of GLT-1 over-expression, 2)
optimal dosing required to provide beneficial effect, 3) verification of the effect of GLT-1
over-expression on supraspinal regions due to pain transmission, 4) and the
demonstration of the anti-nociceptive effect of GLT-1 over-expression in non-human
primates.


Benton RL, Ross CD, Miller KE. Glutamine synthetase activities in spinal white


Galan A, Laird JM, Cervero F. In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons. Pain. 2004 Dec;112(3):315-23.


Herman MA, Jahr CE. Extracellular glutamate concentration in hippocampal


Kaleczyc J, Sienkiewicz W, Klimeczuk M, Czaja K, Lakomy M. Differences in the chemical coding of nerve fibres supplying major populations of neurons between
the caudal mesenteric ganglion and anterior pelvic ganglion in the male pig.

Kamp EH, Jones RC 3rd, Tillman SR, Gebhart GF. Quantitative assessment and
characterization of visceral nociception and hyperalgesia in mice. Am J Physiol
Gastrointest Liver Physiol. 2003 Mar;284(3):G434-44.

Kanai Y, Hediger MA. The glutamate and neutral amino acid transporter family:
31;479(1-3):237-47. Review.

Katano T, Furue H, Okuda-Ashitaka E, Tagaya M, Watanabe M, Yoshimura M, Ito S.
N-ethylmaleimide-sensitive fusion protein (NSF) is involved in central sensitization in
the spinal cord through GluR2 subunit composition switch after inflammation. Eur J

Katano T, Nakazawa T, Nakatsuka T, Watanabe M, Yamamoto T, Ito S. Involvement
of spinal phosphorylation cascade of Tyr1472-NR2B, Thr286-CaMKII, and

Kawamata T, Omote K. Activation of spinal N-methyl-D-aspartate receptors stimulates a
nitric oxide/cyclic guanosine 3,5-monophosphate/glutamate release cascade in

Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K,
Woolf CJ, Ji RR. Ionotropic and metabotropic receptors, protein kinase A, protein
kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response
element-binding protein phosphorylation in dorsal horn neurons, leading to

Kilo S, Schmelz M, Koltzenburg M, Handwerker HO. Different patterns of hyperalgesia

Kohlöffel LU, Koltzenburg M, Handwerker HO. A novel technique for the

Kolhekar R, Gebhart GF. Modulation of spinal visceral nociceptive transmission by

Koltzenburg M, Torebjörk HE, Wahren LK. Nociceptor modulated central sensitization
causes mechanical hyperalgesia in acute chemogenic and chronic neuropathic pain.


Leever JD, Clark S, Weeks AM, Partin KM. Identification of a site in GluR1 and GluR2 that is important for modulation of deactivation and desensitization. Mol Pharmacol. 2003 Jul;64(1):5-10.


McDonald GB, Slattery JT, Bouvier ME, Ren S, Batchelder AL, Kalhorn TF, Schoch


139


Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. Inflamm Bowel Dis. 2010 Nov 8.


Rao VL, Bowen KK, Dempsey RJ. Transient focal cerebral ischemia down-regulates


Santos SD, Carvalho AL, Caldeira MV, Duarte CB. Regulation of AMPA receptors and synaptic plasticity. Neuroscience. 2009 Jan 12;158(1):105-25. Review.


Sheldon AL, Robinson MB. The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention.


Silverman JD, Kruger L. Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. Somatosens Res. 1988;5(3):259-67

Sluka KA, Willis WD. Increased spinal release of excitatory amino acids following intradermal injection of capsaicin is reduced by a protein kinase G inhibitor. Brain Res. 1998 Jul 6;798(1-2):281-6.


Tong CK, MacDermott AB. Both Ca2+-permeable and -impermeable AMPA receptors
contribute to primary synaptic drive onto rat dorsal horn neurons. J Physiol. 2006 Aug 15;575(Pt 1):133-44.


Weng HR, Chen JH, Cata JP. Inhibition of glutamate uptake in the spinal cord induces hyperalgesia and increased responses of spinal dorsal horn neurons to peripheral afferent stimulation. Neuroscience. 2006;138(4):1351-60.


Woolf CJ. What is this thing called pain? J Clin Invest. 2010 Nov 1;120(11):3742-4. Review.


Yaster M, Guan X, Petralia RS, Rothstein JD, Lu W, Tao YX. Effect of inhibition of spinal cord glutamate transporters on inflammatory pain induced by formalin and complete Freund's adjuvant. Anesthesiology. 2011 Feb;114(2):412-23.


