THE ROLE OF GLIAL GLUTAMATE TRANSPORTER GLT-1

OVER-EXPRESSION IN MITIGATING VISCERAL NOCICEPTION

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate School of
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By

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ABSTRACT

Visceral pain is the most common reason for physician visits in US. However, the etiology of visceral hypersensitivity is not clear. Neither is the effective therapy available to relief visceral pain. Glutamate is the major excitatory neurotransmitter and mediates aspects of visceral hypersensitivity. Rapid removal of glutamate from synapse is predominantly mediated by glial glutamate transporter-1 (GLT-1), thus terminating the glutamate stimulation. GLT-1 is found down-regulated in the neuropathic injury model and the artificial induction of GLT-1 attenuates neuropathic pain. In this dissertation, the role of GLT-1 induction in visceral nociception is investigated.

Genetic and pharmacological approaches were utilized to establish GLT-1 induction. In the transgenic mice model, GLT-1 transgene was driven by glial fibrillary acidic protein (GFAP) and over-expressed in astrocyte cells in central nervous system. In the pharmacological model, expression of GLT-1 was enhanced by intraperitoneal administration of ceftriaxone (CTX).

Transgenic animals overexpressing GLT-1 significantly reduced visceral nociceptive responses. In consistent, one week administration of ceftriaxone reduced the visceromotor response (VMR) to colorectal distention (CRD). In addition, this inhibition of visceral nociception could be blocked by systematic treatment with

dihydrokainate (DHK), a selective GLT-1 inhibitor. This data indicates that enhanced glutamate reuptake attenuates colorectal distention-induced visceral nociception.

Further study showed that intrathecal delivery of GLT-1 antagonist DHK to the lumbar spinal cord dose-dependently reversed CTX-blunted visceral nociceptive response, whereas intracisternal delivery of DHK failed to produce visceral anti-nociceptive effects, suggesting a role of spinally located glutamate transporter GLT-1 in visceral nociception. In addition, one-week CTX treatment exhibited no effect on somatic sensory and motor function. This data indicated that GLT-1 upregulation by CTX treatment mitigates visceral nociception through spinal mechanisms.

The role of GLT-1 in animal models of colitis was then investigated. One week CTX treatment upregulated the expression of GLT-1 and reversed the TNBS-induced visceral hypersensitivity. In addition, CTX treatment one week after the onset of DSS-induced visceral inflammation could also attenuate the visceral nociceptive response, suggesting a potential therapeutic effect of CTX. Further experiment revealed that CTX treatment reduced inflammation in the TNBS-induced colitis, but not in DSS model. Cephalothin, another antibiotic lacking GLT-1 induction ability, demonstrated the efficiency to inhibit inflammation but failed to block visceral hypersensitivity, suggesting that GLT-1 over-expression mitigates visceral inflammatory nociception.

Taken together, these studies indicated that GLT-1 over-expression is a novel and effective method to attenuate visceral nociception.

Dedicated to my parents, who have been separated from their only son for more than three years during this dissertation work.

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PUBLICATIONS

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FIELD OF STUDY

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LIST OF SYMBOLS AND ABBREVATIONS

ALS	Amyotrophic Lateral Sclerosis
AMPA	á-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASIC	acid-sensing ion channel
ATP	adenosine triphosphate
BDNF	brain derived neurotrophic factor
CCI	chronic constriction nerve injury
CD	Crohn’s disease
CGRP	calcitonin gene-related peptide
CLT	cephalothin
COX2	cycloxygenase2
CRD	colorectal distention
CREB	cAMP response element binding protein
CRH	corticotrophin releasing hormone
CTX	ceftriaxone
DCN	dorsal column nuclei
DHK	dihydrokainate
DRG	dorsal root ganglia
DSS	dextran sodium sulfate
EAAC1	excitatory amino acid carrier 1
EAAT	excitatory amino acid transporter

EGF	Epidermal growth factor
EMG	electromyography
ERK	extracellular signal-regulated kinase
f-MRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GLAST	glutamate aspartate transporter
GLT-1	glutamate transporter-1
G-protein	guanine nucleotide-binding proteins
GS	glutamine synthetase
HP	hairpin loops
HPA	hypothalamic-pituitary-adrenal
HRP	horseradish peroxidase
HTAB	hexadecyltrimethylammonium bromide
HUGO	Human Genome Organization
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
KCC2	potassium-chloride cotransporter 2
LC	locus coeruleus
mGluR	metabotropic glutamate receptors
MPO	Myeloperoxidase
NE	Norepinephrine
NGF	nerve growth factor

NK1	neurokinin 1
NMDA	N-methyl D-aspartate
NMS	neonatal maternal separation
NOD2	nucleotide oligomerization domain 2
NS	nociceptors specific
ODD	O-dianisidine dihydrochloride
PAG	phosphate-activated glutaminase
PAG	periaqueductal grey
PB	parabranchial nuclei
PGE2	prostaglandin E2
PSDC	postsynaptic dorsal column
RVM	rostral ventromedial medullar
SNL	spinal nerve ligation
SP	substance P
TBOA	Threo-beta-benzyloxyaspartate
TLR	Toll-like Receptor
TM	transmembrane domain
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TRPV	Transient receptor potential gene subfamily V
UC	ulcerative colitis
VGLUT	vesicular glutamate transporter
VMR	visceromotor response
VPL	ventroposteriolateral nucleus
WDR	wide dynamic range

Chapter 1

Introduction

1.1 Glutamatergic neurotransmission in nervous system

1.1.1 Glutamate function and metabolism

Glutamate is one of the twenty standard amino acids that are used for protein synthesis. Its genetic codon is GAA or GAG in DNA sequence. Glutamate is a dicarboxylic amino acid that is essential for cellular metabolism, polypeptide biosynthesis, glutathione synthesis, nitrogen disposal, amino acid transportation and neurotransmission. In humans, glutamate is taken from food, which is broken down by digestion into amino acids. Glutamate is also produced by transamination of the amino group from other amino acids to α-ketoglutarate. In addition, glutamate plays an important role in removing nitrogen. Glutamate is converted to α-ketoglutarate by the enzyme glutamate dehydrogenase (GDH) and produces ammonium. The ammonium is transported to the kidney and is excreted as urea. So, glutamate is an important intermediator to dispose of nitrogen and excrete it from the body.

Glutamate is one of the components required to synthesize glutathione, an important antioxidant to protect cells from oxidative damage. First, intracellular

glutamate is taken by glutamate transporters. The cystine/glutamate antiporter system x(c)(-) then transports extracellular cystine in exchange for intracellular glutamate. Glutamylcysteine is synthesized from glutamate and cysteine via the enzyme gamma-glutamylcysteine synthetase, then glycine is added to the C-terminal of glutamylcysteine via the enzyme glutathione synthetase to produce glutathione. There is evidence suggesting Excitatory Amino Acid Transporters (EAATs) mediate glutamate-dependent enhancement of glutathione synthesis by providing intracellular glutamate and cystine (Rimaniol et al., 2001). Ceftriaxone (CTX), a EAAT2 enhancer, was reported to mediate activation of glutathione through increased nuclear factor erythroid 2-related factor 2 (Nrf2) and system x(c)(-) (Lewerenz et al., 2009).

Glutamate also serves as a precursor to synthesize GABA, an inhibitory neurotransmitter in the brain. GABA is synthesized in GABA-ergic neurons from glutamate by the enzyme glutamate decarboxylase (GAD), which uses pyridoxal phosphate as a cofactor.

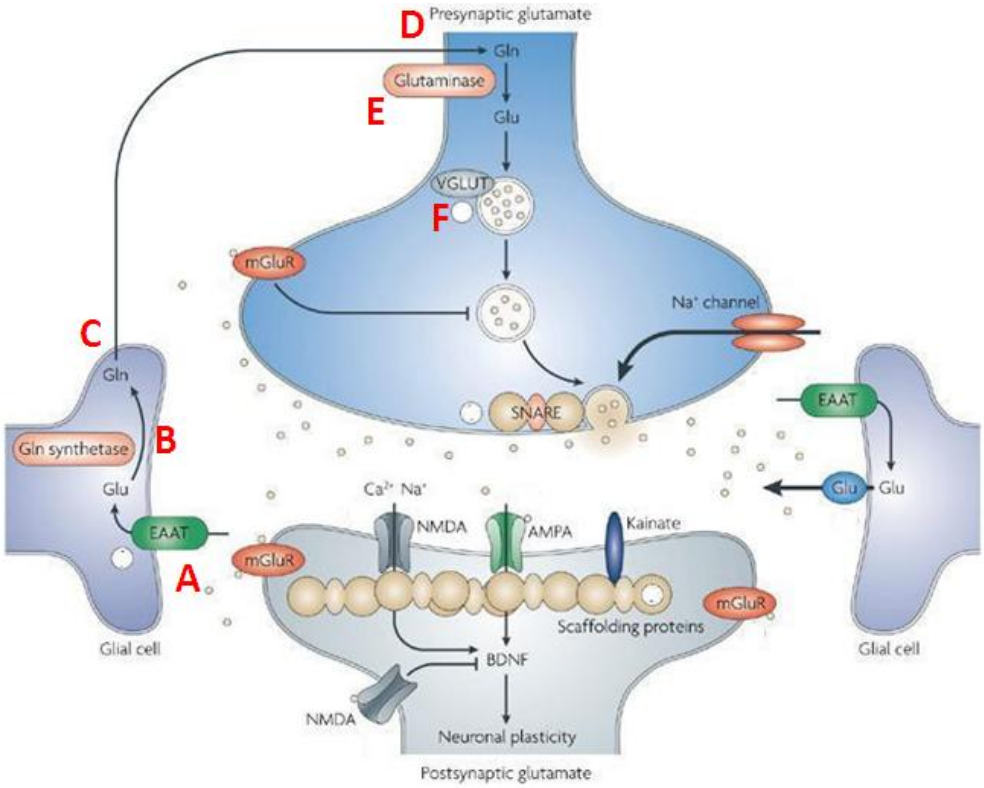
Interestingly, glutamate was also discovered as a flavor enhancer in 1907 by a Japanese scientist Kikunae Ikeda, who created monosodium glutamate (MSG) as a food additive. When tasted, glutamate produces a strong flavor termed as Unami. In the taste buds of the tongue, glutamate binds to its receptor, a modified form of mGluR4, and triggers subsequent signals, which involve releasing the neurotransmitter ATP to nearby nerve terminals (Nelson et al., 2002).

1.1.2 Glutamate as neurotransmitter

Glutamate is considered to be the most dominant excitatory neurotransmitters in the brain and is involved in most aspects of brain function including perception and movement, learning and memory (Headley et al., 1990). Glutamatergic neurons exist in more than 50% of brain tissue and are most abundant in the cerebral cortex and hippocampus, with projections to all parts of the brain (Headley et al., 1990). The average concentration of glutamate in human brain tissue is 5-15 mmol/Kg, and the extracellular concentration of glutamate by dialysis measurement is 1-4 μM (Lerma et al., 1986) while the intracellular concentration of glutamate is around 2.5-10 mM (Nicholson et al., 1996), with the highest concentration found in nerve terminals. The significant gradient between intracellular and extracellular glutamate concentration is maintained by high-affinity glutamate transporters, especially the excitatory amino acid transporters (EAATs), in the plasma membrane. The function of the glutamate transporter is highly dependent on cell energy supply and glutamate may leak out of the cell in case of energy failure (Danbolt, 2001). Since there is no enzyme to degrade glutamate in the extracellular space, a high concentration of extracellular glutamate can quickly become very toxic. This hyper-glutamatergic excitotoxic state will over activate NMDA glutamate receptors and induce degeneration of neurons.

In glutamatergic neurotransmission, action potentials in the presynaptic terminal lead to membrane depolarization and opening of voltage-gated Ca channels. Ca influx triggers a series of events including the docking of glutamate-containing

vesicles to the presynaptic active zones and the fusion of these vesicles to the plasma membrane. Glutamate is released into the synaptic cleft by exocytosis and diffuses to activate the glutamate receptors on the postsynaptic terminal. Glutamate stimulates two broad classes of receptors: ion channel linked ionotropic receptors and metabotropic receptors (Tanabe et al., 1992). Activation of different postsynaptic glutamate receptors triggers a series of responses, including generation of action potentials and modification of synaptic characteristics. The glutamate excitatory action is terminated by the quick reuptake of glutamate from the synaptic cleft to nearby glia or neurons, which is mediated by the sodium-dependent high-affinity glutamate transporter.



(**Figure 1.1 Glutamatergic neurotransmission**. Adapted from Sanacora et al., 2008)

There is a mechanism that exists to reuse glutamate after its uptake into glia, known as the glutamate-glutamine cycle (Figure 1.1 A) (Daikhin et al., 2000). In astrocyte glial cells, glutamate is converted into glutamine by glutamine synthetase (GS) (Figure 1.1 B). Glutamine is then released into the extracellular space by system N transporter (Figure 1.1 C) and is taken up into nearby neurons by system A transporter (Figure 1.1 D) (Chaudhry et al., 2002). In neurons, glutamine is deaminated to glutamate by the enzyme phosphate-activated glutaminase (PAG) (Figure 1.1 E) and transported into presynaptic vesicles by vesicular glutamate transporter (VGLUT) (Figure 1.1 F), thus completing the whole cycle of glutamate reuptake (Fremeau et al., 2004).

1.1.3 Glutamate receptors

Glutamate receptors are transmembrane receptors that are responsible for glutamate mediated excitatory neurotransmission. There are two major classes of glutamate receptors: ion channel linked ionotropic receptors and G-protein coupled metabotropic receptors (mGluR). The ionotropic glutamate receptors include ion channels that open upon the presence of glutamate and mediate fast glutamate responses, whereas the metabotropic glutamate receptors activate guanine nucleotide-binding proteins (G-protein) that trigger intracellular secondary messengers and mediate slow glutamate responses.

There are different subtypes of ionotropic and metabotropic glutamate receptors in neurons and glia. The ionotropic receptors are divided into three subtypes based on the specific agonists: the *N*-methyl *D*-aspartate (NMDA) receptor, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the kainate receptor. Metabotropic receptors include eight subtypes, named mGluR1 to mGluR8.

The NMDA receptor is the most studied neuronal glutamate receptor because of its important role in synaptic plasticity and learning and memory (Fei et al., 2009). The NMDA receptor is a heterotetramer composed of two NR1 and two NR2 subunits which are transmembrane proteins and form an ion channel together. Glutamate binds with high affinity to the NR2 subunits of the NMDA receptor while glycine binds as a co-agonist to the NR1 subunits. High diversity exists in the NR1 protein, due to different alternative splicing of GRIN1 mRNA (Stephenson et al., 2006). In vertebrates, NR2 subunits contain four distinct isoforms, named NR2A to NR2D. So, the NMDA receptor structure is quite different in the different cell types and developmental stages (Liu et al., 2004). Besides this, the long intracellular domain of each subunit binds to a series of adaptors and protein kinases. Taken together, it explains the complexity of the NMDA receptor. Another characteristic of the NMDA receptor is its voltage dependent activation and calcium permeability. During the resting period, the ion channel of the NMDA receptor is often blocked by magnesium, even in the presence of glutamate. When the synaptic membrane is depolarized to -30 mV, due to the activation of the AMPA receptor, the magnesium leaves its binding site and the ion channel is opened,

which allows calcium influx into the cell (Nowak et al., 1984). Increasing calcium concentration is a key process to trigger subsequent signaling pathways and leads to the changes in synaptic properties, termed synaptic plasticity (Dingledine et al., 1999). Generally, NMDA receptors containing NR2A or NR2B subunits generate 'high-conductance' channel openings with a high sensitivity to Mg^{2+}, whereas NR2C- or NR2D-containing receptors exhibit 'low-conductance' openings with a lower sensitivity to extracellular Mg^{2+} (Momiyama et al., 1996). However, Ca^{2+} permeability of NMDAR channels is not significantly modulated by NR2 subunit composition (Schneggenburger et al., 1996). Excitotoxic neuronal death caused by excessive calcium influx through NMDA receptor is involved in stroke and seizure (Lee et al., 1999).

The AMPA receptor is responsible for the quick, voltage-independent glutamate response in the synapse. The AMPA receptor is a tetramer composed of two GluR1 and two GluR1 or GluR3 or GluR4 subunits (Mayer et al., 2005). Each of the subunits contains a glutamate binding site in the extracellular domain and allows sodium influx through the ion channel which leads to the depolarization of the membrane. One characteristic of the AMPA receptor is the rapid desensitization and channel closure after opening, due to conformational changes (Armstrong et al., 2006). By this mechanism, the AMPA receptor is considered to trigger most of the fast glutamatergic transmission in the brain. The permeability of the AMPA receptor to calcium is dependant on the presence of the GluR2 subunit (Hollmann et al., 1991). The AMPA

receptor is permeable to calcium in the absence of the GluR2 subunit, since GluR2 directly blocks calcium influx. The calcium permeable AMPA receptor is able to enhance LTP function and plasticity (Youn et al., 2008). The long intracellular C terminal domain of the AMPA receptor can be activated by phosphorylation and interact with downstream adaptor proteins by PDZ domain (Boehm et al., 2006). The abnormal AMPA receptor is involved in many neurological diseases, such as epilepsy and traumatic brain injury (Platt et al., 2007).

The kainate receptor is less understood than the NMDA and AMPA receptors. It is a tetramer composed of five different subunits: GluR5-7, KA1 and KA2. GluR 5-7 exhibit less than 40% homology with the AMPA receptor subunits GluR1-4 and do not co-assemble with these subunits (Cui et al., 1999). Cells from different brain regions express distinct kainate receptor subunits with distinct pharmacology and electrophysiology properties (Wilding and Huettner, 2001). Ion channel properties of kainate receptors are similar to the AMPA receptors in that they mediate fast glutamate response and are permeable to sodium and potassium (Huettner et al., 2003). Its distribution and synaptic function is more limited than other receptors. The mGLuR5-7 mRNAs of kainate receptors undergo diverse alternative splicing in the cytoplasmic C terminals, affecting their trafficking and activity (Schiffer et al., 1997). The kainate receptor is involved in seizures and excitotoxicity (Mulle et al., 1998).

The metabotropic glutamate receptors are membrane associated G-protein coupled receptors. They mediate indirect secondary messenger pathways and are involved in the

modulation of synaptic activity (Hinoi et al., 2001). There are eight subtypes of mGluRs, labeled as mGluR1 to mGluR8, which are divided into three groups based on structure and function. mGluR1 and mGluR 5 form group Ⅰ, which activates phospholipase C and leads to inositol 1,4,5-trisphosphate (IP3) induction and calcium channel opening (Endoh et al., 2004). Group Ⅱ receptors include mGluR2 and mGluR3, while group Ⅲ receptors include mGluR4, 6, 7, 8. Both of the group Ⅱ and group Ⅲ receptors inhibit adenylyl cyclase and reduce secondary messenger cyclic adenosine monophosphate (cAMP) (Endoh et al., 2004). Besides the difference in function, different groups of mGluRs have distinct cellular locations. Most of the mGluR Ⅰ receptors are located in the presynaptic membrane, whereas most of the mGluR Ⅱ receptors are located in the postsynaptic membrane (Shigemoto et al., 1997).

1.1.4 Glutamate uptake

Glutamate transporters are responsible for the clearance of extracellular glutamate, after its release into the synapse. It is well known that there is a significant difference between intracellular and extracellular glutamate concentrations and scientists had hypothesized the existence of a potent glutamate transport system in the brain for a long time. However, little was known about this glutamate uptake system, until the successful cloning of Excitatory Amino Acid Transporter (EAAT) gene family in the 1990s (Danbolt, 2001). These findings revealed that the EAATs are sodium dependent high affinity glutamate transporters which mediate the majority of glutamate transport

across the plasma membrane, which will be mentioned in detail in section 1.2 (Danbolt, 2001). Besides transport across the plasma membrane, there is another glutamate uptake system that exists in the presynaptic terminal, which transports glutamate from the cytoplasm to glutamatergic vesicles so that glutamate can be released into synaptic cleft. This function is executed by vesicle glutamate transporters (VGluTs), a protein family that is totally distinct from the EAATs and is dependent on a proton gradient (Naito et al., 1983).

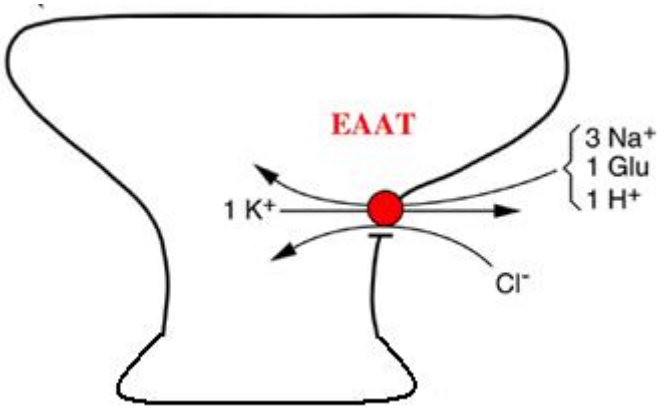
1.2 Excitatory Amino Acid Transporter (EAAT)

1.2.1 Structure and function of EAAT family

There have been five types of EAAT genes characterized, termed EAAT1-5. In rodents, EAAT1, EAAT2 and EAAT3 are also named GLAST, GLT and EAAC1, respectively, as the original discoverers named them in the early 1990s. In humans, the EAAT gene family is also commonly named the SCL1 gene family, according to the official Human Genome Organization (HUGO) (Kanai et al., 2004). All of the EAATs share 50-55% homology with each other. There is 98% homology between rodent and human EAAT homologs. Based on a study of a bacterial EAAT homolog, which shares 37% sequence identity with human EAAT2 (Yernool et al., 2004), EAATs are eight-transmembrane (TM) domain proteins with two hairpin loops (HP) in the C terminal (Figure 1.3) (Kanner et al., 2006). TM1-6 are conventional alpha helix domains,

whereas TM7 contains two unique re-entrant loops in close proximity. In the membrane, the EAATs form a homotrimetric complex by non-covalent interaction, which contains a characteristic bowl and a solvent-filled basin that extend half-way across the membrane (Gether et al., 2006). The glutamate-binding site is located in TM8 and the sodium- and potassium-binding sites are located in HP2 and TM7, respectively.

Glutamate transport is driven by the potassium-sodium gradient across the cell membrane. Determination of EAAT3 coupling stoichiometry reveals that one glutamate is cotransported with three sodium ions and one proton and is countertransported with one potassium ion (Zerangue et al., 1996). Other EAATs share similar transport stoichiometry with EAAT3 (Figure 1.2).

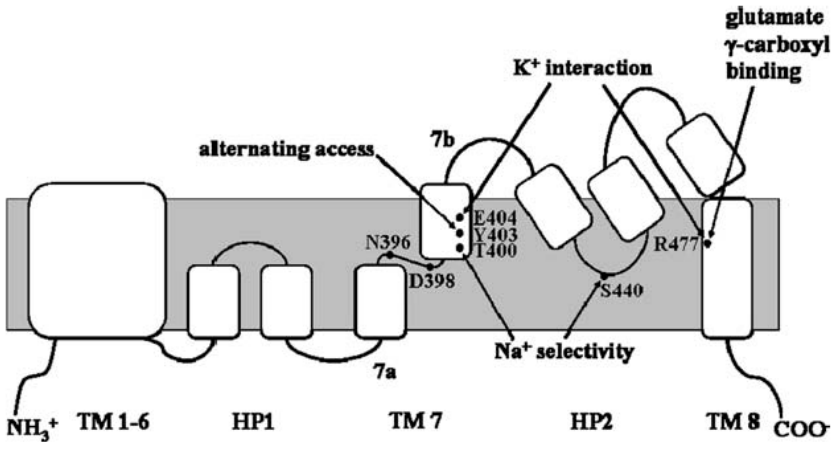


(**Figure 1.2 Schematic for EAAT stoichiometry**. adopted from Danbolt, 2001)

Recently, the crystal structure of the bacterial homologue of EAAT2 was analyzed and indicated that the HP1 and HP2 domains in TM7 function as the intracellular and extracellular gates (Reyes et al., 2009). The structure of this transporter can be partitioned into two large domains: a trimerization domain which includes TM1, 2, 4, 5

and a transport domain which includes TM3, 6, 7, 8. Binding of glutamate and sodium to EAAT2 leads to the closure of intracellular and extracellular gates, and then isomerization occurs upon movement of the transport domain relative to the trimerization domain, which leads to the opening of the inward facing gate and the release of glutamate (Reyes et al., 2009).

However, in some special circumstances, such as ischemia, cell energy failure leads to reversed glutamate transport through EAAT (Kauppinen et al., 1988). After stroke, deprivation of oxygen causes the shortage of cell energy and the disturbance of ion balance across the membrane. In this case, extracellular potassium rises and intracellular sodium decreases, so that the electrochemical gradient is not able to support glutamate uptake and results in the release of intracellular glutamate through the EAAT and subsequent excitotoxicity (Rossi et al., 2000).



(Figure 1.3 The structure of EAAT2 protein. Adapted from Kanner et al., 2006)

1.2.2 EAAT1

The rodent homolog of the EAAT1 gene was identified from rat brain in 1992 and named the glutamate aspartate transporter (GLAST) (Stock et al., 1992). The 3 kb EAAT1 cDNA encodes a 66-kD glycoprotein with 543 amino acid residues. Human EAAT1 protein shares 96% sequence identity with rat GLAST. In situ hybridization reveals high expression of EAAT1 mRNA in astrocytes and Bergmann glia cells in the cerebellum and less dense distribution in the cerebral cortex and brain stem (Figure 1.4 A). The EAAT1 expression is not restricted in the glia cells, it was also found in ependymal cells, meningeal cells and tanycytes in the CNS (Berger et al., 2000). Outside of the CNS, EAAT1 expression has been discovered in cochlear neurons and bone marrow cells (Furness 1997, Mason 1997).

EAAT1 is a specific transporter for glutamate and aspartate with a proposed stoichiometry of three sodium, one glutamate and one potassium (Kloeckner et al., 1993). EAAT1 reaches saturated kinetics at 77 ± 27 μM glutamate, and shows no significant transport of other amino acids including alanine, leucine, glutamine and arginine. The loss of EAAT1 has been demonstrated in neurodegenerative disease. For example, aberrant expression of EAAT1 has been reported in Alzheimer's disease (Scott et al., 2002).

EAAT1 knockout mice were generated in 1998. The research revealed a moderate deficit in motor coordination, although the mice developed normally (Watase et al., 1998). The knockout mice also showed increased susceptibility to cerebellar injury.

These results are consistent with dysfunction of the cerebellum and the abundant expression of EAAT1 in the cerebellum.

1.2.3 EAAT2

The rodent homolog of the EAAT2 gene was identified from rat brain in 1992 and named the glutamate transporter 1 (GLT1) (Pines et al., 1992). The EAAT2 gene encodes a 62.1kD protein with 573 amino acid residues and eight transmembrane domains. It is a glycoprotein with two glycosylation sites and two phosphorylation sites. Human EAAT2 shares 95% sequence identity with rat GLT1. Different isoforms of EAAT2 proteins were reported with variation in the C terminal (Chen et al., 2002). A mutation (N206S) in the EAAT2 gene was found in ALS cases, in which the asparagine in residue 206 is replaced by a serine (Aoki et al., 1998).

In situ hybridization reveals high level expression of EAAT2 in the cerebral cortex and hippocampus and relative low expression in the cerebellum and brain stem. In the spinal cord, EAAT2 is expressed higher in the grey matter than that of GLAST (Rothstein et al., 1994) (Figure 1.4 B). EAAT2 expression is found in all astrocytes, with higher expression in the membranes facing synapses (Chaudhry et al., 1995). There is evidence showing neuronal expression of EAAT2 mRNA in the CA3 pyramidal neurons and cerebral cortex layer Ⅴ neurons, whereas neuronal protein expression of EAAT2 protein was only found during early development (Sutherland et al., 1996). The discrepancy between mRNA and protein levels in neurons is a puzzle

and may be due to the 11kb EAAT2 mRNA structure with a long 3'UTR sequence (Pines et al., 1992). Outside of the CNS, EAAT2 is also found in small intestine, liver, lymph nodes, spleen and pancreas (Iwanaga et al., 2005).

EAAT2 is the most physiologically important transporter for glutamate. Transport studies show that EAAT2 is responsible for more than 90% of glutamate uptake in the brain (Danbolt, 2001). Quantitative studies demonstrate that EAAT2 density is up to 8500 molecules per μm^2 in the hippocampus glia cell membrane, which is four times higher than GLAST density in the hippocampus (Lehre et al., 1998).

The importance of EAAT2 in brain function is also demonstrated by EAAT2 knockout animals. The homozygous mice lacking EAAT2 are viable but exhibit increased exacerbation of brain injury and lethal epileptic seizures in adulthood (Tanaka et al., 1997). Selective neuronal degeneration is also observed in the hippocampus CA1 region. Electrophysiological recording in hippocampus slices reveals significantly less inhibition of EPSCs by NMDA receptor antagonists in knockout mice than controls, while normal decay of EPSCs is not altered (Tong et al., 1994).

In many neurological disorders, loss of EAAT2 has been associated with increased susceptibility to brain damage. In Amyotrophic Lateral Sclerosis (ALS), the specific loss of EAAT2 is found in both sporadic ALS patients and a mutant SOD1 transgenic mouse model of ALS (Fray et al., 1998; Bendotti et al., 2001). The mechanism of loss of EAAT2 may be due to post-transcriptional regulation by

oxidative stress, because mRNA levels of EAAT2 remain the same (Bristol et al., 1996). In ischemia, disturbance of ion balance resulting from energy failure reverses the glutamate transport through EAAT2 and kills nearby neurons by glutamate-induced excitotoxicity (Rossi et al., 2000). In Alzheimer's disease (AD), the loss of EAAT2 protein but not mRNA is also reported in AD patients and in mutant APP transgenic mice with memory deficit (Li et al., 1997; Mesliah et al., 2000). However, it is still not clear whether A β 42, a toxic peptide found in AD, can directly cause the loss of EAAT2 expression (Butterfield et al., 2002; Rodriguez-Kern et al., 2003). In animal models of pathological pain states, including chronic constriction nerve injury and spinal nerve ligation, both EAAT1 and EAAT2 protein expression are increased immediately after surgery and then attenuated 5-7 days postoperatively (Sung et al., 2003; Binns et al., 2005).

1.2.4 EAAT3

The rodent homolog of the EAAT3 gene was identified from rat small intestine in 1992 and named the excitatory amino acid carrier 1(EAAC1) (Kanai et al., 1992). It encodes a 57kD protein and share 92% sequence identity with human EAAT3 protein. EAAT3 is widely located in the cell body and presynaptic terminals of neurons in the forebrain and cerebellum, which is in sharp contrast to the location of EAAT1 and EAAT2 (Rothstein et al., 1994) (Figure 1.4 C). The distinct expression pattern suggests EAAT3 not only functions as a glutamate transporter, but also serves other

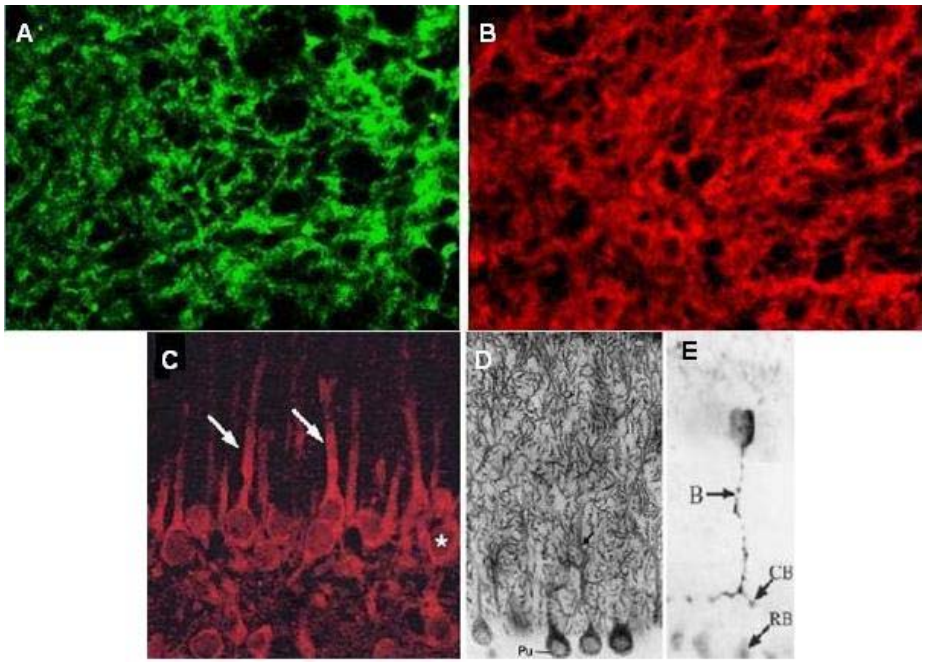
cellular function in the nervous system. EAAT3 is capable of transporting cystine, as well as glutamate, quickly into the neurons, both of which are precursors to the synthesis of glutathione, an important antioxidant in the brain (Aoyama et al., 2006). EAAT3 knockout mice develop normally, however, with reduced neuronal glutathione level and exhibit neurodegeneration and behavior deficits during aging (Aoyama et al., 2006). This phenomenon indicates a critical role of EAAT3 in the brain oxidative metabolism.

1.2.5 EAAT4

The EAAT4 gene was cloned from human cerebellum in 1995 (Fairman et al., 1995). EAAT4 shares 65%, 41%, 48% sequence identity with human EAAT1, 2 and 3, respectively. EAAT4 is primarily located in the cerebellar Purkinje cells with little expression in the forebrain (Figure 1.4 D). Subcellular analysis reveals EAAT4 is located in the cell body and dendritic spines (Tanaka et al., 1997). EAAT4 has similar pharmacological property to other glutamate transporters with Km (Glu) = 3.3±0.4 μM (Fairman et al., 1995). The unique characteristic of EAAT4 is the permeability to chloride and functions as a ligand gated chloride channel (Fairman et al., 1995). Therefore, EAAT4 is able to modify Purkinje cell excitability by enhancing chloride influx (Palmada et al., 1998). EAAT4 knockout mice exhibits larger amplitude and faster rising kinetics in mGluR mediated EPSCs (Nikkuni et al., 2007).

1.2.6 EAAT5

The EAAT5 gene was cloned from salamander and human retina in 1997 (Arriza et al., 1997). The EAAT5 gene encodes a 560 amino acid protein and shares 64%, 36%, 37%, 43% sequence identity with human EAAT1, 2, 3 and 4. EAAT5 is primarily located in rod, cone and rod bipolar cells in the retina (Wersinger et al., 2006) (Figure 1.4 E). Similar to EAAT4, EAAT5 is also permeable to chloride (Arriza et al., 1997). The activation of EAAT5 is sodium and voltage dependent and lead to chloride influx. It is also noteworthy that the C terminal end of EAAT5 contains PDZ domains, which suggests EAAT5 can function as a signaling molecule (Sheng et al., 1996). These interesting properties make EAAT5 an inhibitory presynaptic receptor in rod bipolar cells and outline its importance in visual processing (Wersinger et al., 2006).



(**Figure 1.4. The expression of EAATs.** (A) EAAT1 in the spinal cord. (B) EAAT2 in

the spinal cord. (C) EAAT3 in the hippocampus CA1. (D) EAAT4 in the cerebellum. (E) EAAT5 in the retina. B: bilopar cell, CB: cone bipolar cell, RB: rod bipolar cell. Adapted from Xin et al., 2009; He et al., 200; Dehnes et al., 1998; Pow et al., 2000)

1.3 Visceral pain

1.3.1 Introduction to pain

Pain is a common symptom in medicine. However, the etiology of chronic, persistent pain is not yet completely understood. According to the American Academy of Pain Medicine, pain is an unpleasant sensation and an emotional response to that sensation. It explains that pain is not only a nociceptive perception, but also an unpleasant emotional response that involves higher cognitive functions (Price et al., 1980). In clinical practice, pain is defined as "whatever the experiencing person says it is, existing when he says it is" (McCaffery et al., 1968).

Because of its uncertain nature, the history of pain research is full of debate. In ancient Greece, Aristotle (384-322 BC) first proposed that pain is an affect and the heart is the seat of feeling. At a similar time, the ancient Chinese canon of medicine, the *Huang Di Nei Jing* (2000-250 BC), states that pain is evoked by the disturbance of an internal signal, termed "Chi", in a network inside the human body and is connected to the heart. Later, the Roman physician Galen (AD 130-201) indicated the brain as the master organ of sensation based on animal experiments. Little progress was made in the

Middle Ages until 1664 when Rene Descartes described a pain pathway from skin to brain and proposed peripheral nerves as the media for pain transport. In the early nineteenth century, Charles Bell and Francois Magendie found that the spinal dorsal root ganglia are sensory in function (Bell, 1811). In 1894, based on a historical quantitative experiment, Max von Fray proved that pain is a special sensation, instead of an emotion or intensive concomitant of other sensations (von Fray, 1894). In 1906, Charles Sherrington further proposed the concept of nociceptor, which is a specific receptor responding to noxious and potentially damaging stimuli (Sherrington et al., 1906). In the most recent one hundred years, with the development of electrophysiology and molecular biology tools, the detailed pain pathways and pain receptor molecules were revealed.

The current theory of pain states that pain is generated by activation of nociceptors and is transmitted by pain-transmitting afferents through peripheral nerves to the spinal cord or brain stem and is finally projected to sites mediating pain perception in the brain. Based on the different types of painful stimuli, pain can be divided into several categories: mechanical pain, thermal pain, inflammatory pain and neuropathic pain. Pain can also be classified into somatic pain and visceral pain, by the different locations of stimuli. Visceral pain displays distinct symptomatic characters and neuroanatomic pathways from somatic pain (Chapter 1.3.2).

Several nociceptors have been discovered in recent decade, which are capable to detect thermal, mechanical and inflammatory stimuli. Transient receptor potential gene

subfamily V, member 1 (TRPV1) was cloned in 1997 (Caterina et al., 1997). TRPV1 is a nonselective cation channel that is activated by both chemicals such as capsaicin, mustard oil and the heat greater than 43°C. The activation of TRVP1 generates painful burning sensation. Two years later, TRPV2, a homologue of TRPV1, was cloned and was indicated to response to the heat greater than 52°C (Caterina et al., 1999). Other TRP family nociceptors were identified later on. TRPV3 is activated by the heat between 22 and 40°C (Peier et al., 2002a). TRPV4 is activated by the heat greater than 25°C, cell swelling, mechanical and thermal stimuli (Vriens et al., 2004). TRPM8 is activated by the cool temperature lower than 23°C and by a cooling agent, menthol (Peier et al., 2002b) TRPA1 is activated by the cold temperate lower than 17°C , pungent compounds and mechanical stimuli (Story et al., 2003; Kwan et al., 2006). The discovery of molecular basis of nociception in the recent decade has strongly supported the theory that pain is a specific sensation and greatly improved our understanding of the nature of pain.

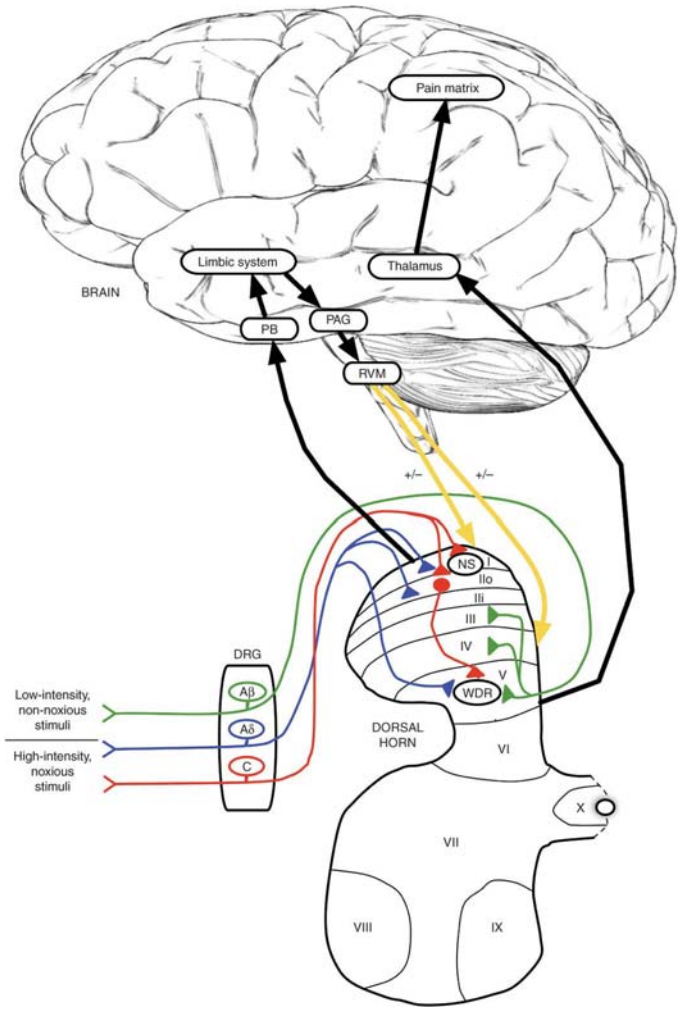
Some nociceptive pathways have been identified. In the peripheral nervous system, Aδ and C fibers transmit impulse after the activation of nociceptors in the periphery and carry nociceptive signals to the central nervous system (D'Mello et al., 2008) (Figure 1.5). C fibers are unmyelinated small-diameter nerves with a relatively slow conduction velocity (2m/s). Aδ fibers are moderately myelinated medium-diameter nerves with a relatively higher conduction velocity (20m/s). Both of the Aδ and C fibers have cell bodies in the dorsal root ganglia (DRG) and project from the peripheral terminals to the

first central neurons in the superficial layers of spinal cord dorsal horn. There is another type of nociceptor called silent nociceptor, which normally are unresponsive to noxious stimuli but only become responsive to stimuli after inflammation or tissue injury.

In the spinal level of central nervous system, there are six laminae in the dorsal horn of the spinal cord (Rexed et al., 1952) (Figure 1.5). Lamina Ⅰ is the marginal layer and lamina Ⅱ is known as *substantia gelatinosa* with a translucent appearance because of lack of myelin. Lamina Ⅰ and Ⅱ form a clear superficial layer of spinal cord and receive small diameter fiber input with nociceptive information. Lamina Ⅲ, Ⅳ, Ⅴ and Ⅵ, contain *nucleus proprius* and make up the deep dorsal horn region with less organized neurons and afferent input. Aβ fibers that connect to low threshold mechanoreceptors terminate in lamina Ⅲ and its border with lamina Ⅱ and is generally considered to bring non-nociceptive sensory, whereas Aδ fibers that connect to high threshold mechanoreceptors terminate in lamina Ⅰ and Ⅴ and are responsible for nociceptive sensory function(Light et al., 1979). The unmyelinated thin C fibers are specific for nociception and temperature and project mostly to lamina Ⅱ with some terminations in lamina Ⅰ and Ⅲ (Sugiura et al., 1986). There are different types of sensory neurons in the spinal cold dorsal horn. Massive numbers of small interneurons are found in all area of superficial dorsal horn (Malmberg et al., 1997). These interneurons are either excitatory in function with expression of glutamate and PKC-γ, or inhibitory in function with expression of GABA (Malmberg et al., 1997). The large projection neurons are mostly located in lamina Ⅰ, Ⅲ, Ⅳ and Ⅴ. They use glutamate

as excitatory transmitter and often co-express substance P (SP) and calcitonin gene-related peptide (CGRP) (Brown et al., 1995). Large spinal neurons are divided into three classes by their electrophysiological properties. Two major classes of spinal projection neurons are responsible for nociceptive processing: wide dynamic range (WDR) neurons and nociceptors specific (NS) neurons. WDR neurons are known as class 2 neurons and located in the deep dorsal horn region. They exhibit large cell bodies with extensive dendrite and response to broad range of intensity of the peripheral stimuli. NS neurons are mainly found in the lamina Ⅰ and known as Waldeyer's neurons or class 3 neurons. They have relatively small size and restricted response to nociceptors from the skin, muscle and viscera (Cervero et al., 1995). Class 1 neurons exhibit low threshold to peripheral stimuli and are generally considered to be non-nociceptive neurons.

In the supra-spinal level of central nervous system, the axons of nociceptive-specific spinal neurons travel across over the anterior white commissure to the contralateral side of the spinal cord and go up to ventroposteriolateral nucleus (VPL) of the thalamus via the lateral spinothalamic tract, also known as the anterolateral system (Figure 1.5). Some of these ascending fibers also synapse in brainstem, such as rostral ventromedial medullar (RVM) and parabranchial (PB) nuclei (D'Mello et al., 2008). The third-order neurons in the thalamus project to cortical regions, including insular cortex, anterior cingular cortex and somatosensory cortex, which generate the perception of pain (Craig et al., 2003).



(**Figure 1.5 Schematic somatic ascending and descending pain pathway**. NS, nociceptive-specific neuron; WDR: wide dynamic range neuron; DRG, dorsal root ganglion; PB, parabranchial nuclei; PAG, periaqueductal grey nuclei; RVM, rostral ventromedial medullar nuclei. Adapted from D'Mello et al., 2008.)

On the other hand, there are descending pathways existing for pain modulation, which exhibit both facilitatory and inhibitory natures (Figure 1.5). The descending inhibitory projection travels from brainstem nuclei including locus coeruleus (LC) and periaqueductal grey (PAG) nuclei to the primary afferent terminals in the spinal cord

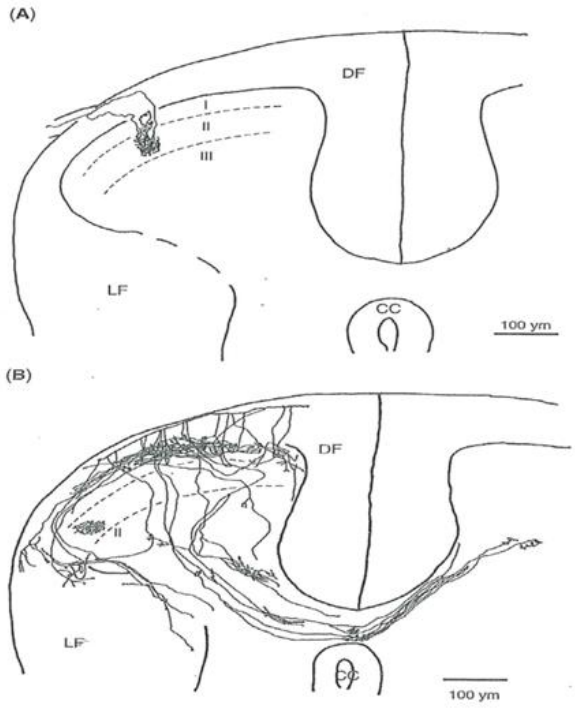
dorsal horn. Norepinephrine (NE) is involved in this inhibitory pathway and function on alpha2 adrenoceptors in the spinal neurons to suppress firing rate (Millan et al., 2002). The descending facilitatory pathway travels from brainstem nuclei including RVM and raphe nuclei down to the superficial dorsal horn. Serotonin is involved in this facilitatory pathway and activates 5-HT3 receptor positive spinal neurons (Green et al., 2000). This facilitatory pathway contributes to spinal central sensitization and enhances nociceptive response in neuropathic pain (Suzuki et al., 2004).

1.3.2 Visceral pain

Nociception can be classified into somatic pain or visceral pain, by the different locations of nociceptive stimuli. Visceral pain is generated from visceral organs located inside thoracic and abdominal cavities including heart, gastrointestinal tract and urinary tract, whereas somatic pain evokes from skin and superficial tissues. Pain emanating from bone, muscle, joint, blood vessel, ligament and tendons is termed deep somatic pain.

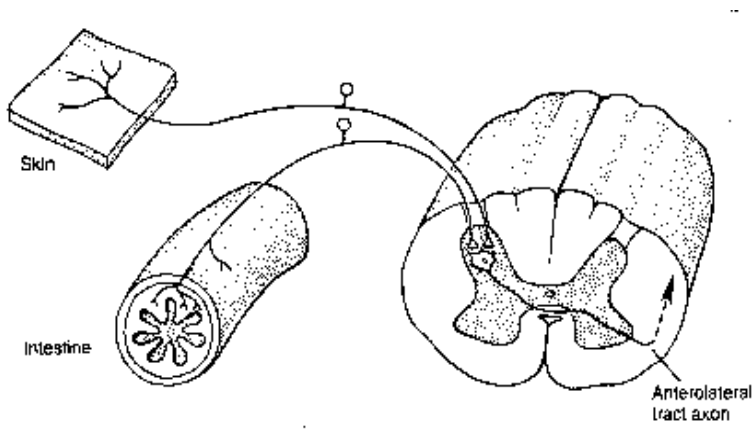
Visceral pain is a common form of pain produced from internal organs and is the most frequent reason patients visit their doctors (Cervero et al., 1999). However, visceral pain is much less understood than somatic pain, because our knowledge about pain derives mostly from research in somatic pain (Cervero et al., 1999). More importantly, the nature of visceral pain is distinct from that of somatic pain in the following five specific characteristics. (1) Visceral pain is not evoked from all viscera,

as some viscera are lack of afferent innervations. Many diseases of the liver, lungs or kidney are painless (Meyrier et al., 1993). (2) Some injury to visceral organs does not cause pain; for example, cutting the intestine does not cause pain. (3) Visceral pain is diffuse and difficult to localize, because visceral nerves have extensive branches terminating in the viscera and in the spinal cord (Figure 1.6). (4) Visceral pain is often referred to the somatic cutaneous site in the body because of the convergence of the visceral nerve and the somatic nerve in the spinal cord (Figure 1.7). (5) Visceral pain is linked with enhanced motor and autonomic reflexes, including nausea and vomiting (Joshi et al., 2000). These phenomena suggest unique neurobiology mechanisms of visceral pain that are distinctly different from those associated with somatic pain.



(**Figure 1.6 Peripheral afferents termination in the spinal cord**. Visceral afferents

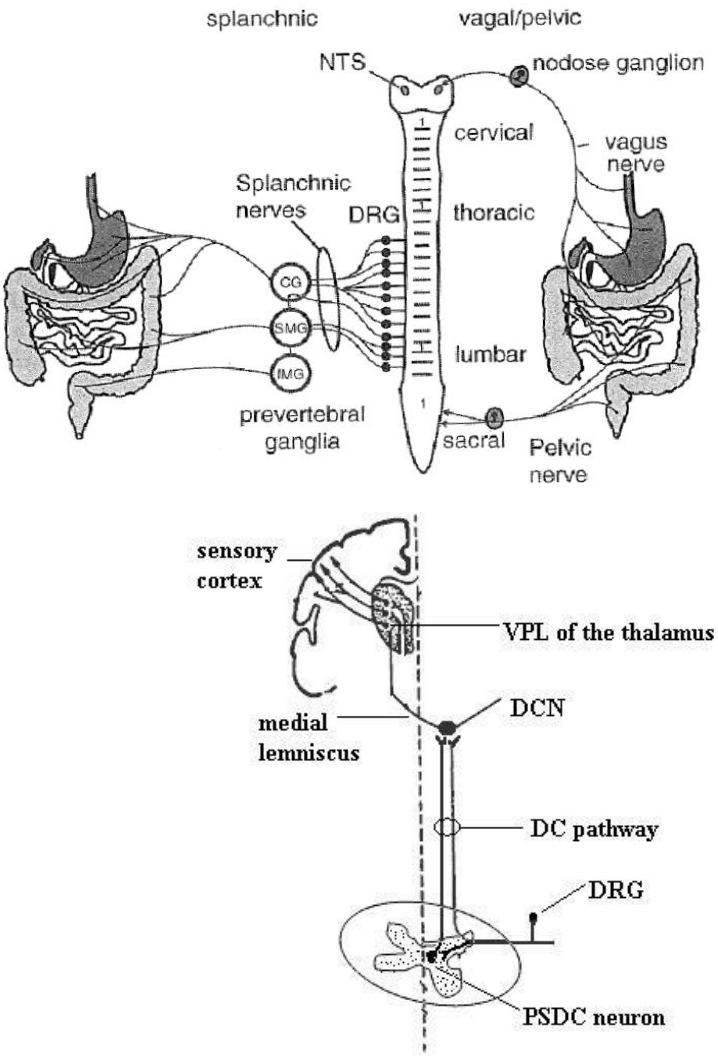
exhibit extensive branches terminating in the spinal cord (B), compared to somatic afferents do (A).)



(**Figure 1.7 Illustration of somatic-visceral convergence for referred pain**. Two DRG neurons separately innervating skin and intestine converge to a single spinal neuron.)

Visceral nociception utilizes different ascending pathway in both peripheral and central nervous system, compared with somatic nociception (Figure 1.8). The visceral nociceptive afferent typically travels along sympathetic and parasympathetic nerves to the first central neurons in the spinal cord. Similar to the somatic afferent, the visceral nociceptive afferent is composed of either unmyelinated C fiber or thinly myelinated Aδ fiber with cell body in the spinal and cranial ganglia. The peripheral terminals of visceral afferent are located in mucosa, muscle, mesentery and serosa (Lynn et al., 1999). In the spinal cord, the visceral afferents have both extensive ascending and descending branches and terminate diversely in lamina Ⅰ, Ⅱ, Ⅳ, Ⅴ, X, and

occasionally in the dorsal and lateral funiculi and contralateral lamina V and X (Sugiura et al., 1989). It is estimated that 50%-70% of the spinal dorsal horn neurons response to visceral stimulation, whereas only 2%-10% of the afferent fibers response to visceral stimulation. The discrepancy may due to the extensive arborization of the visceral fibers in the spinal cord (Fig 1.6), which accounts for cross-talk between visceral-somatic fibers and the poor localization of visceral pain (Sugiyura et al., 1989). Besides the laternal spinothalamic tract, some visceral nociceptive-specific neurons in the lamina X of spinal cord also project through dorsal column pathway to the gracile and cuneate nuclei in the brainstem, which is distinct from somatic nociception transmission (Willis et al., 1999). In the higher level, the visceral nociceptive nerves reach cortical nociceptive region, such as the insula and anterior cingulate cortex, and limbic system (Cechetto et al., 1987).



(**Figure 1.8 Schematic of peripheral (upper) and central (lower) visceral pain pathway.** Adapted from Wang 2008. PSDC ,postsynaptic dorsal column; VPL ,ventral posterolateral; DRG ,dorsal root ganglion; DC pathway, dorsal column pathway; DCN, dorsal column nuclei.)

The majority of the visceral pain fibers are mechanosentitive and polymodal in character (Su et al., 1998). For example, 100% of pelvic nerves respond to 1g von Fray hairs (Brierley et al., 2004). Study from the mechanical visceral sensory system

indicates three different sensory receptors in the viscera (Cervero et al., 1994). The first type of receptors shows a high threshold to nociceptive stimuli. The second type of receptors shows a low threshold to stimuli and an intensity encoding function. The third receptors, which are also termed silent nociceptors, do not respond to the normal stimuli and are only activated in case of tissue damage or inflammation. So far, several nociceptor molecules belonged to the TRPV family have been identified in the visceral organs. The expression of TRPV1, TRPV4 and TRPA1 are located in the GI tract. The selective deletion of the above genes leads to a reduced response to colon distention (Blackshaw et al., 2010). Particularly, TRPV4 knockout mouse exhibits an increased mechanosensitivity threshold (Brierley et al., 2008). Increased TRPV1 expression in the nerve fibers, mast cell and lymphocytes was found in the Irritable Bowel Syndrome (IBS) patients (Akbar et al., 2008). In addition, acid-sensing ion channel 3 (ASIC3) knockout mice also showed less sensitive to colon distention (Jones et al., 2007).

Glutamate is an important excitatory neurotransmitter in the visceral pain pathway (Sengupta et al., 2009). The release of glutamate in the spinal synapse leads to the activation of AMPA and NMDA receptor. In disease status, such as colonic inflammation, the NR1 subunit of NMDA receptor is upregulated (Li et al., 2006). NMDA receptor antagonists reduce acid-induced chemical hyperalgesia (Willert et al., 2004) and colon distension-induced mechanical hyperalgesia (Traub et al., 2002). Most of the visceral sensory fibers contain neuropeptides, such as substance P, suggesting a specific role of substance P in the visceral hyperalgesia (Cervero et al., 1999).

Application of neurokinin receptor antagonists SR140333 and SR48968 reduces visceral hyperalgesia (Kamp et al., 2001). The inhibitory neurotransmitter GABA is involved in antinociception, mainly via the activation of GABAb receptor in the inhibitory inter-neurons in spinal cord (Brusberg et al., 2009). 5-HT (Serotonin) is widely expressed in the GI tract and is of particular importance in visceral hypersensitivity (Coldwell et al., 2007). Toxic stimuli triggers the release of 5-HT from endocrine cells of the gut. The activation of 5-HT3 and 5-HT4 receptor in the visceral afferents could lead to visceral hypersensitivity (Hicks et al., 2002).

Visceral pain is one of the most common symptoms in patients suffering Irritable Bowel Syndrome (IBS). IBS is a bowel disorder with chronic abdominal pain, increased gut permeability and motility alteration. The prevalence rate for IBS is between 8% and 20% in US population with a ratio of 2:1 in females and males (Muller et al., 2001). The etiology of IBS is unknown, since most of the IBS patients are lack of detectable pathology and abnormality, such as tissue damage or inflammation. Neither is the effective therapy available to IBS patients. Alosetron and tegaserod, which are selective 5-HT3 antagonist and 5-HT4 agonist respectively, are approved by FDA to reduce visceral pain in IBS. Several hypotheses were proposed for the pathogenesis of IBS, including the gut inflammation, gut flora disturbance, serotonin dysregulation in the gut, dysfunction of brain-gut axis and stress (Stark et al., 2007). Further understanding of the mechanism of visceral pain would contribute to the effective therapy for IBS.

1.3.2 Animal models of visceral pain

Although visceral pain is very common in the clinic, there is still not a fully acceptable animal model to mimic visceral pain and to investigate therapeutic methods. In rodents, the study of visceral nociception is mostly approached by the distention of the hollow viscera, such as colon, bladder, stomach and uterus, at graded pressures and measured by the abdominal muscle contraction response in the form of electromyography (EMG). Colorectal distention (CRD) is a commonly employed visceral pain model, which is caused by direct stimulus to the colon by an inflated balloon. CRD-induced abdominal muscle contraction, also termed visceromotor response (VMR), could be measured by the EMG electrodes implanted in the external oblique muscle as an index of visceral nociception, since VMR is reduced dose dependently by morphine (Kamp et al., 2003). VMR is such an easily accessed and controlled method that it replaces conventional methods measuring respiratory rate and cardiovascular parameter as visceral pain index. However, VMR is a pseudo-affective response and reflects only a small portion of nociceptive response (Wang et al., 2008 c). In addition to EMG measurement, CRD-induced perigenual cingulate cortex activation, which is not activated by somatic nociception, can be monitored by functional magnetic resonance imaging (fMRI) method to measure visceral nociception (Derbyshire et al., 2007). fMRI detects different paramagnetic properties of oxy- and deoxyhaemoglobin

in the cerebral blood vessel, thus indirectly providing regional neuronal activity information (Sharma et al., 2009). The advantage of fMRI is excellent spatial resolution (2-5mm) and non-invasive monitor, whereas its limitation is poor temporal resolution (1-3s) and lack of neurotransmitter information (Sharma et al., 2009).

Colonic inflammation/irritation models of visceral pain consist of local injection of inflammatory chemicals or neonatal stress. The inflammation model is the most common method to produce visceral hyperalgesia. The immune response triggers the release of cytokines, TNF-alpha, ATP and histamine, which subsequently sensitize nociceptors in the colon and produces visceral hypersensitivity (Bielefeldt et al., 2009). Colonic injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS) induces colitis and damage of colonic mucosa, which is due to T cell mediated immuno-response against hapten modified autologous proteins (Wirtz et al., 2007). The symptoms of TNBS-induced colitis include dense infiltrations of the lymphocytes/macrophages and thickening of the colon wall (Neurath et al., 1996). Local zymosan administration establishes a brief colitis or bladder inflammation. Zymosan binds to Toll-like Receptor (TLR2) that induces NF-kappaB activation and TNF-alpha secretion, resulting in inflammation (Sato et al., 2003). Interestingly, zymosan-induced visceral hypersensitivity lasts for several weeks, even after inflammation symptoms disappear (Jones et al., 2007). Intracolonic instillation of oxazolone, a hapten reagent, causes severe colitis with weight reduction, diarrhea and marked loss of goblet cells (Boirivant et al., 1998). The oxazolone -induced inflammation only affects the mucosa of the distal

colon and can be attenuated by anti-IL-4 antibody. Colitis can also be produced by adding dextran sodium sulfate (DDS) into drinking water (Okayasu et al., 1990). DSS can directly damage the epithelial cell in the colon and induce CD4 cell activation (Wirtz et al., 2007). Please refer to chapter 4 for more information about TNBS- and DSS- induced colitis.

Stressful experience in the early life, including physical, sexual and emotional abuse and a life threatening situation, has been linked to a subset of IBS patients (Chitkara et al., 2008). This observation leads to the development of a neonatal maternal separation (NMS)-induced visceral hypersensitivity model in the absence of visceral inflammation. In rodents, 180 min maternal separation in P2 to P14 pups is sufficient to induce visceral hypersensitivity in adulthood (Coutinho et al., 2002). The mechanism proposed was that maternal separation could regulate long term activity of hypothalamic-pituitary-adrenal (HPA) axis, leading to decreased corticotrophin releasing hormone (CRH) and enhanced glucocorticoid activity (Liu et al., 1997). The activation of the HPA axis would have a profound effect on sensory transmission in the spinal cord by increasing NGF activity and modulating descending pathways (Chung et al., 2007). The stress and HPA activation not only triggers central sensitization in the spinal cord, but also affects visceral inflammation through mast cells. The maternal separation enhances mast cell density and degranulation and the release of inflammatory factors in the gut (Barreau 2007).

In addition to the neonatal maternal separation model, neonatal noxious stimulus on

colon, bladder or somatic referred site could also give rise to chronic visceral hypersensitivity. Repetitive colon distention at 60 mmHg or repetitive intracolonic administration of mustard oil in P8 to P21 rat pups leads to allodynia in adulthood (Al-chaer et al., 2000). Both of the central sensitization of spinal neurons and the peripheral sensitization of primary visceral afferents are present in the neonatal noxious stimuli-induced visceral hypersensitivity. However, the same stimuli in the postnatal day 21 or 45 do not induce the visceral hypersensitivity. Similarly, intravesicular instillation of zymosan in P14 to P16 female rat pups results in hyperalgesia and frequent micturition in adulthood (Randish et al., 2006). The hyperalgesia in this neonatal bladder inflammation model is not only seen in response to the bladder distention, but also to the colon distention, which could be explained by the cross organ sensitization. Furthermore, neonatal stimulus in gastrocnemius muscle, a somatic referred site of colon, is capable of producing hyperalgesia to colon distention in adulthood, which is due to somatovisceral convergence in the spinal cord (Miranda et al., 2006).

1.4 The role of glutamate in pain physiology

1.4.1 Glutamate-induced activity-dependent central sensitization

Central sensitization is the functional enhancement of the pain-transmitting neurons in the spinal dorsal horn in response to intensive noxious stimuli, inflammation or nerve injury. Altered neuronal plasticity leads to a reduction in pain threshold, an

increased response to noxious stimuli, a spread of receptive field and an enhanced spontaneous activity (Latremoliere et al., 2009). Central sensitization could be applied to explain the mechanism of abnormal pain sensitivity even without obvious pathology (Latremoliere et al., 2009).

Central sensitization was first demonstrated in 1983 when electrophysiological analysis from spinal motor neurons in the flexor reflex revealed increased excitability of alpha motor neurons after repetitive noxious heat stimuli (Woolf et al., 1983). Later, central sensitization was discovered in spinal sensory neurons (Cook et al., 1986; Woolf et al., 1988). Further investigation supported the idea that this activity-dependent change in nociceptive transmission is evoked from alterations within the spinal cord, instead of the periphery. Large low-threshold Aβ fibers, which elicit no pain response in the normal condition, produce pain response to low intensity input after repetitive noxious stimuli (Woolf et al., 1983). Descending inhibitory modulation in the spinal cord is attenuated (Traub et al., 1997). The current theory states that central sensitization is induced by increase in membrane excitability, synaptic efficacy and loss of inhibition (Latremoliere et al., 2009).

Glutamate and its receptors play critical roles in the molecular mechanism of central sensitization. The activity-dependent central sensitization is composed of two phases: the early post-translational dependent phase and the late transcription dependent phase. The repetitive noxious stimuli cause prolonged membrane depolarization and the activation of glutamate NMDA receptors (Woolf et al., 1991).

The opening of the NMDA receptors allows entry of calcium into neurons and activates intracellular PKC and MAPK signaling pathways (Chen et al., 1992), which lead to the phosphorylation of ERK, an important downstream protein kinase (Ji et al., 1999). Activation of ERK has a profound effect on neuronal plasticity. When activated, ERK phosphorylates the NR1 subunit of the NMDA receptor and enhances its response to glutamate by increasing the opening of the receptor channel (Zou et al., 2000). The elevated ERK activity also results in the increased AMPA receptors expression in the postsynaptic membrane by promoting the trafficking of the AMPA receptors (Zhu et al., 2002). In addition, ERK leads to the phosphorylation of Kv4.2, a voltage gated potassium channel, and therefore increases the membrane excitability (Hu et al., 2003). The above modulations occur quickly and explain the mechanism of the early phase of central sensitization. The mechanism of the late phase of central sensitization involves transcriptional regulation. The phosphorylation of ERK activates cAMP response element binding protein (CREB) and other transcriptional factors. The activated CREB translocates into nucleus and enhances the expression of many synapse-related genes, including c-Fos, NK1, TrkB and Cox2, which result in the long term sensitization of postsynaptic neurons (Ji et al., 1997).

Glutamate is also involved in another form of synaptic facilitation termed wind up. Wind up is a phenomenon of the increased neuronal firing rate in response to low-frequency (0.5-5Hz) C fiber stimuli. Wind up is a result of cumulative membrane depolarization, which leads to the generation of action potentials when it reaches the

threshold (Mendell et al., 1965). After the stimulation, the terminals of C fibers release glutamate, as well as substance P (SP) and calcitonin gene related peptide (CGRP) into the first central synapse within the spinal cord (Murase et al., 1986). The continuous activation of NK1 and CGRP1 receptors cause a cumulative long-lasting membrane depolarization, which activates NMDA receptors by removing magnesium from the blocking site. The activation of NMDA receptors further depolarizes the membrane and finally induces action potential (Morisset et al., 2000). Wind up is distinct from the activity-dependent central sensitization, although the stimuli that induce both of them could be similar. Wind up is a homo-synaptic facilitation, which can only last for several seconds and disappear when membrane potential returns back to normal (Ji et al., 2003). Whereas, central sensitization involves multiple synapses and lasts for at least several hours. The heterosynaptic property of the central sensitization is responsible for the generation of secondary hyperalgesia, a spread of pain beyond the injury site (Ji et al., 2003).

1.4.2 Central sensitization in somatic chronic pain

Central sensitization is not only involved in the activity-dependent synaptic enhancement, but also contributes to the chronic pain hypersensitivity, including inflammatory pain and neuropathic pain. The molecular mechanism of central sensitization in chronic pain is similar to that of activity-dependent central sensitization with its own characteristics.

In inflammatory pain, tissue damage leads to the release of inflammatory mediators and nerve growth factor (NGF) in the periphery and alters the input of sensory afferents (Woolf et al., 1996). The large non-nociceptive DRG neurons begin to release SP and BDNF into the spinal cord in response to the non-noxious stimuli (Neumann et al., 1996). The intensity of the Aβ fiber-mediated synapse is significantly increased from very low level baseline (Baba et al., 1999). The induction of cycloxygenase2 (Cox2) in the spinal dorsal horn neurons is a key marker in the inflammatory pain (Samad et al., 2001). Cox2 mediates the increase of prostaglandin E2 (PGE2), which binds to its EP2 receptors in the postsynaptic membrane. The EP2 receptor is a G-protein coupled receptor, which facilitates the ion channel function of the glutamate NMDA and AMPA receptors and increases the membrane excitability (Kohno et al., 2008). NMDA receptor NR2B subunit is phosphorylated by Src after inflammatory injury (Guo et al., 2002). The AMPA receptors also undergo conformational change by replacing the GluR2 subunits with the GluR1 subunits that are calcium permeable (Park et al., 2009; Larsson et al., 2008; Vikman et al., 2008). Activated microglia due to inflammation also contributes to the central sensitization by the activation of MAPK pathway and the release of the pro-inflammatory cytokines, such as IL-1β and TNF-α (Svensson et al., 2003).

In neuropathic pain, nerve injury generates spontaneous firing of the afferent fibers, activates massive numbers of glial cells and immune cells, and results in the profound gene expression changes in the DRG neurons (Latremoliere et al., 2008). The synthesis

of SP, BDNF and NOS are greatly enhanced. In particular, peripheral nerve injury results in sprouting of Aβ fiber from non-nociceptive neurons to nociceptive-specific neurons, which switch innoxious stimuli to noxious stimuli (Woolf et al., 1992). In addition, ATP activates microglia via the P2X4 receptor and mediates the release of BDNF from the microglia (Tsuda et al., 2003). BDNF disturbs the expression of KCC2, a K-Cl cotransporter, in the GABA-ergic neurons and inverts the inhibitory effect of GABA, resulting in the loss of inhibitory modulation and the enhanced central sensitization in the spinal cord (Coull et al., 2005).

1.4.3 Central sensitization in visceral pain

Central sensitization plays an important role in visceral pain. IBS patients exhibit hypersensitivity not only limited to the GI tract, but also to other sites leading to back pain, migraine and heartburn, suggesting broad alteration in central pain processing (Verne et al., 2001). An animal model of visceral hypersensitivity shows that neonatal colonic irritation leads to chronic visceral hypersensitivity associated with central sensitization in the adult animals (Al Chaer et al., 2000). These rats also display hypersensitivity to cutaneous nociceptive stimuli with absence of colon pathology (Wang et al., 2008 b). Electrophysiology studies reveal that prolonged noxious stimuli in the viscera change the receptive field and increase the excitability of visceral neurons in the spinal cord (Cervero et al., 1995). The increase of central neuron excitability is mediated by spinal sensitization and descending facilitation from the brain (Moshiree et

al., 2006).

Evidence suggests that glutamate acting at NMDA receptors is essential for enhanced visceral hypersensitivity, since administration of NMDA receptor antagonist ketamine reduces central neuron excitability and inhibits the visceral nociceptive response (Cervero et al., 1995). The expression of NMDA receptor NR1 subunit splice variants is increased after TNBS-induced colitis, which is a key to maintain visceral hypersensitivity (Zhou et al., 2009).

Central sensitization may explain the pathogenesis of referred pain (Moshiree et al., 2006). Essentially, referred pain is a secondary hyperalgesia resulting from the central sensitization. The mechanism of the viscero-somatic referred pain is the synaptic convergence of both somatic and visceral afferents onto a single dorsal horn neuron in the spinal cord (Moshiree et al., 2006) (Figure 1.7). The phosphorylation of NMDA receptor NR2B subunit by Src kinase leads to enhanced visceral hypersensitivity and cross organ sensitization (Peng et al., 2010).

1.4.4 Glutamate transporter in central sensitization and pain

The role of glutamate transporters in pain is not well understood. Altered expression of glutamate transporters have been found in neuropathic pain. In a neuropathic pain model, chronic constriction nerve injury (CCI) induces an early-phase upregulation of EAAT1, EAAT2 and EAAT3 in the postoperative day 1 and 4, which is followed by a late-phase downregulation of these transporters in the postoperative day 7 and 14 (Sung

et al., 2003; Xin et al., 2009). The similar result also exists in the spinal nerve ligation (SNL) model of neuropathic pain (Wang et al., 2008 a). The downregulation of EAAT2 is also associated with chemotherapy-induced mechanical hyperalgesia (Weng et al., 2005).

NF-kappaB is an important signaling pathway to regulate EAAT2 expression. Epidermal growth factor (EGF) activates NF-kappaB and induces EAAT2 expression (Sitcheran et al., 2005). An antibiotic drug ceftriaxone (CTX) also stimulates EAAT2 expression via NF-kappaB activation (Lee et al., 2008).

Interestingly, the enhanced spinal expression of EAAT2 by either viral transgene infection or intrathecal CTX injection does not alter acute somatic pain, including mechanical pain and thermal pain (Maeda et al., 2008; Hu et al., 2009). However, the enhanced spinal expression of EAAT2 attenuates hypersensitivity in both neuropathic pain and inflammatory pain (Maeda et al., 2008; Hu et al., 2009). These results suggest a potential important role of EAAT2 in attenuating chronic pain. Further study of EAAT2 revealed that the inhibition of EAAT2 by specific blocker, DHK, leads to the prolonged excitatory postsynaptic currents (EPSC), which could be mediated by presynaptic inhibition, desensitization of the postsynaptic AMPA receptors and glutamate spillover (Weng et al., 2007). Another nonselective glutamate transporter blocker, TBOA, results in the increased expression of NR2B subunit of the NMDA receptors and the enhanced response of the NMDA receptors (Nie et al., 2009). This project explains the role of increased glutamate uptake in mitigating visceral

nociception.

Chapter 2

Increased glia glutamate transporter GLT-1 expression reduces visceral nociceptive response in mice

2.1 Abstract

Visceral pain is the most common reason for physician visits in the US (Christianson et al., 2009). The etiology of visceral hypersensitivity is not clear. Glutamate is the major excitatory neurotransmitter and mediates aspects of visceral hypersensitivity (Lynn et al., 1992). GLT-1 is the predominant glutamate transporter in the central nervous system, mediates 90% of glutamate reuptake activity and thus terminates glutamatergic transmission (Danbolt, 2001). In the present study, the function of GLT-1 in an animal model of visceral pain was investigated. The data showed that transgenic animals overexpressing GLT-1 significantly reduced visceral nociceptive responses. Moreover, one week administration of ceftriaxone, a specific GLT-1 enhancer, reduced the visceromotor response (VMR) to colorectal distention (CRD). In addition, this inhibition of visceral nociception could be blocked by systematic treatment with dihydrokainate (DHK), a selective GLT-1 inhibitor. The data indicates that enhanced glutamate reuptake attenuates colorectal distention-induced

visceral nociception.

2.2 Introduction

Irritable bowel syndrome (IBS) is a group of idiopathic bowel disorders in which abdominal discomfort or pain is associated with defecation or a change in bowel habit, and with features of disordered defecation (Thompson et al., 1999). The clinic diagnostic criteria for IBS are the presentation of two of the following three features in 12 weeks of one year: relieved with defecation, onset associated with a change in frequency of stool or onset associated with a change in form of stool (Thompson et al., 1999). The prevalence of IBS is between 8% and 20% in US and western countries and is greater in women than men (Drossman et al., 2002). The onset of IBS symptoms is between 30-50 years old with a decline in older subjects. However, it is estimated that the majority of people with IBS symptoms do not consult physicians. The pathological symptoms of IBS include: (1) abnormal GI motility in stomach, small intestine, colon, and rectum; (2) increased visceral hypersensitivity as measured by balloon distention in the bowel; (3) altered gut immune function; (4) abnormalities in extrinsic autonomic innervations of the viscera and (5) alteration in brain-gut communication (Drossman et al., 2002). In addition to physiological changes, psychological factors also affect IBS. IBS patients are more susceptible to stress-induced GI dysfunction, compared to healthy controls. Furthermore, IBS patients report more stress events, such as severe

childhood abuse history, which are associated with disease onset and severity (Drossman et al., 1996). Because of the unknown etiology, medications are only available for symptomatic treatment of IBS, such as altered gut function and depression. These medications include dietary therapy, antispasmodics and anticholinergic reagents, 5-HT antagonists and antidepressant drugs (Drossman et al., 1996). Particularly, alosetron and tegaserod, which are selective 5-HT3 antagonist and 5-HT4 agonist, respectively, are approved by the FDA to treat IBS.

Commonly employed visceral pain models include i.p. injection of irritants and balloon distention of the distal colon. The injection of 0.6% acetic acid into the abdomen induces a characteristic writhing response over a short time span. It is a simple method to measure visceral pain by counting the numbers of writhes. However, algesia induced by this method can not be solely attributable to visceral organ, as a somatic component may also be presented (Christianson et al., 2007). Besides, these irritants are not natural stimuli and not well controlled by investigator. Colorectal distention (CRD) is a more visceral-specific method and involves direct stimulation of the colon by balloon inflation. CRD-induced abdominal muscle contraction, also termed the visceromotor response (VMR), is measured by electromyography (EMG) electrodes implanted in the abdomen. The VMR is considered an index of visceral nociception, since VMR is reduced in a dose dependent manner by morphine (Kamp et al., 2003) and blocking TRPV1 can blunt visceral hypersensitivity (Jones et al., 2007) . CRD has been applied to the colorectum in rodents and the descending colon in other

animals (Christianson et al., 2007). The CRD model is the most commonly applied animal model stimulating visceral nociception, largely because the experiment parameters, such as stimulation position, balloon size, pressure and duration of distention, can be easily controlled by the investigator and the VMR can be reliably quantified using the EMG in awake animals. The visceromotor response is intensity dependent and temporally associated with the onset and termination of stimulation (Christianson et al., 2007). However, the VMR is a pseudo-affective response that reflects only a small portion of the nociceptive response (Wang et al., 2008 c).

Glutamatergic transmission is involved in visceral nociception. Intravenous administration of the NMDA receptor antagonist ketamine prevented acid-induced esophageal hypersensitivity in human subjects (Willert et al., 2004). Spinal administration of NMDA facilitated the neuronal response to CRD (Kolhekar et al., 1996). In an animal model of colitis, the NR1 subunit of NMDA receptor was up-regulated, phosphorylated in the myentric plexus (Zhou et al., 2006) and underwent alternative splicing in the spinal cord (Zhou 2009). Compared to glutamate receptors, little is known about the putative role of glutamate transporters in mediating visceral pain. In contrast, the role of the glutamate transporter in mediating neuropathic pain is better understood. In the partial sciatic nerve ligation-induced neuropathic pain model, the glutamate transporters GLAST and GLT-1 were found initially increased at 1 day and then down regulated 5 days after nerve injury (Sung et al., 2003). In the CFA-induced inflammatory pain model, virus infection of the GLT-1 transgene attenuated the

inflammatory pain response in the hind paw (Maeda et al., 2008). The over-expression of the glutamate transporter GLT-1 by ceftriaxone, a beta lactam antibiotic, significantly reversed the mechanical and thermal allodynia caused by nerve injury (Hu et al., 2009). One week administration of ceftriaxone has been well established to produce GLT-1 over-expression (Rothstein et al., 2005). CTX targets an unknown receptor in astrocytes and triggers an unknown intracellular signaling leading to the activation of NF-kappaB. The P65 component of NF-kappaB is released and translocated into the nucleus upon disassociation with the I-kappaB component. P65 then binds to the kappaB responding element in the promoter region of the GLT-1 gene and stimulates GLT-1 transcription (Lee et al., 2008).

The present study describes a significant reduction of visceral nociception in GLT-1 overexpressing transgenic animals and animals receiving one week CTX treatment. GLT-1 transgenic mice, which showed a two-fold increase in glutamate reuptake, exhibited a significant reduction in visceromotor response to colorectal distention. Moreover, systemic pretreatment with the selective GLT-1 blocker DHK reversed the CTX-blunted visceral nociception.

2.3 Methods

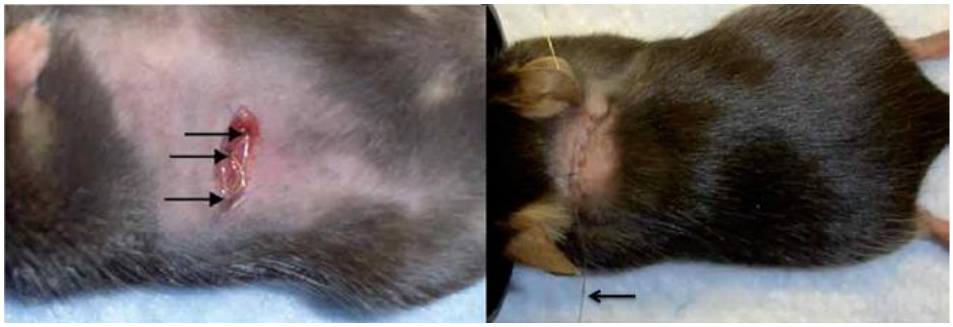
Animals. Two to three-month old FVB/N mice weighing between 20 and 33 grams were used in this study. These mice were obtained from Harlan (Indianapolis, IN) and

were housed in groups of five with free access to water and food. All protocols were approved by the Institutional Animal Care and Use Committee at the Ohio State University and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

GLT-1 transgenic mice. Transgenic mice overexpressing human GLT-1 protein were kindly provided by Dr. Glenn Lin, Ohio State University. The 1.8 kb human GLT-1 cDNA was driven by an astrocyte-specific 2.2kb-long glial fibrillary acidic protein (GFAP) promoter with a c-Myc tag in the N-terminal and a mouse protamine-1 (MP1) gene 3' untranslated region in the C-terminal. The transgene was microinjected into FVB/N mice fertilized eggs. The genotype of the transgenic mice was determined by PCR on the genomic DNA extracted from tail biopsies and transgene- specific primers (5_-ggc aac tgg gga tgt aca-3_ and 5_- gac ggc agc atc cta gaa agg t -3_). PCR protocol was as follows: 95°C for 3 min, 85°C for 2 min; 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for 30 cycles followed by 10 min extension at 72°C.

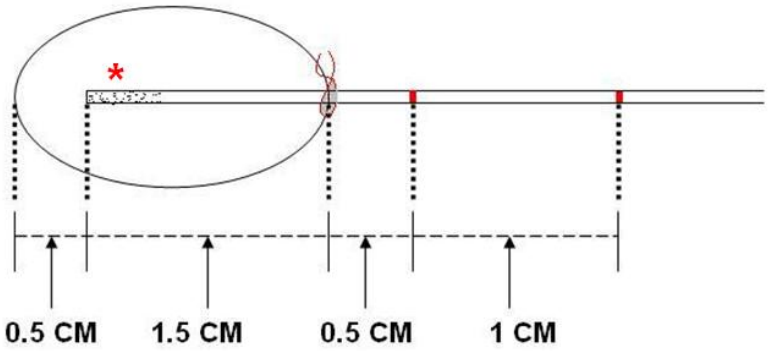
CRD surgery. Mice were anesthetized by intraperitoneal administration of ketamine (137mg/Kg, Hospira) and xylazine (20mg/Kg, Bayer). Hair was removed by a shaver in the lower right abdomen and the back of the neck. The exposed skin was disinfected by 0.75% iodine and 75% ethanol. Two small 1-cm-long incisions were made in the back neck and the lower right abdomen rostral to the inguinal ligament. One 30-cm-long

EMG electrode wire was made from Teflon-coated wires (Cooner wire, CA) and the coating in the terminal 0.5 cm of both ends was stripped. One end of the stripped wire was then assembled into a 25G needle which was inserted into the external oblique muscle rostrally. The needle was removed later and the stripped electrode tip was fixed through the muscle layer by a knot of suture (6-0 silk). The same procedure was repeated to place the other end of the stripped wire in the abdominal muscle about 0.5 cm from the first one. As a result, a loop of wire was formed toward the tail of the mouse in the abdomen. A small surgical clamp was then inserted from the neck incision all the way subcutaneously to the abdominal incision and the loop was grabbed back to the neck incision by the clamp. The abdominal incision was closed by three 7.5-mm-wide Michael surgical clips. The electrode wire in the neck was secured to the superficial muscle of the neck by a knot of suture. To avoid the loss of wire after surgery, the wire left in the neck was placed under the skin of the back with 1.5 cm loop out. Another knot of suture was made to secure the wire tip and the neck incision was closed by two Michael clips. Finally, a 1.5-cm-long loop in the neck was cut and two separate electrodes were implanted completely. 0.1-ml warm saline was administrated to animals intraperitoneally after surgery to compensate the loss of fluids. Animals were single housed and allowed three days to recover from the surgery.



(**Figure 2.1 Illustration of CRD surgery.** Left, arrow indicates the implanted abdominal EMG wire. Right, arrow indicates EMG wire left in the neck (adapted from Christianson et al., 2007).)

CRD balloon construction. Balloons used in colorectal distention were made by assembling polyethylene plastic to PE-60 tubing. Several holes were punched in the distal 0.5 cm end of a 10 cm PE-60 tubing to facilitate gas flow into the balloon. A piece of 3-cm x 3-cm polyethylene plastic was stretched against a 1-cm x 1-cm rod to form a 2.5-cm-long cylinder. The PE-60 tubing was inserted 2-cm deep into the plastic cylinder and the end of the cylinder was secured tightly to the tubing by a knot of suture. The extra piece of plastic and suture silk was removed and one small drop of glue was placed at the site of the knot to prevent the leakage of gas from the balloon. Two markers were labeled in the tubing 0.5 cm and 1.5 cm away from the knot. The first marker indicated the location of the anus and the second one was the check point during the test. A 4-cm-long protective sheath, made by cutting PE-240 tubing lengthwise, was used to cover the balloon during rectal insertion and was removed after insertion.



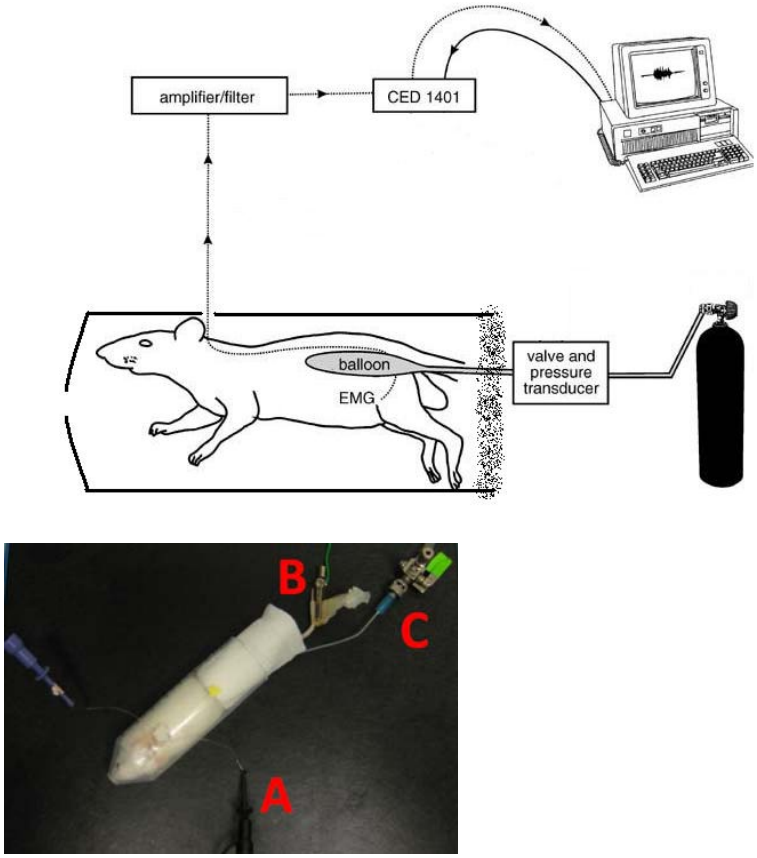
(**Figure 2.2 Diagram for CRD balloon construction.** The tip of a PE-60 tube was punched with several holes (asterisk), sealed to a plastic balloon (red loop) and labeled at the end (red markers, left marker indicates the position of anus).)

Animal restraint devices. Restraint devices were constructed from a 50 ml plastic conical tube (Fisher Scientific, Hanover Park, IL). The plastic tube was cut in a 90º angle at the 40-ml mark by a heated blade. The wall of tube was cut lengthwise down leaving a 2-cm gap on the ventral aspect and a 2-cm-wide glass slide was glued to the ventral aspect of the tube, providing a flat surface for better acclimation of the mice in the tube. A small 0.5-cm x 0.5-cm hole was cut in the top of the tube for access to the EMG electrode. The internal diameter of the tube was approximately 2.5-cm, which was capable of holding a 20- to 30-g mouse (Figure 2.3).

Balloon distention. Animals were fasted one day before the test with free access to water to reduce defecation during CRD. Animals were lightly anesthetized by 4% isoflurane induction followed by 1.5% isoflurane maintenance in the anesthesia

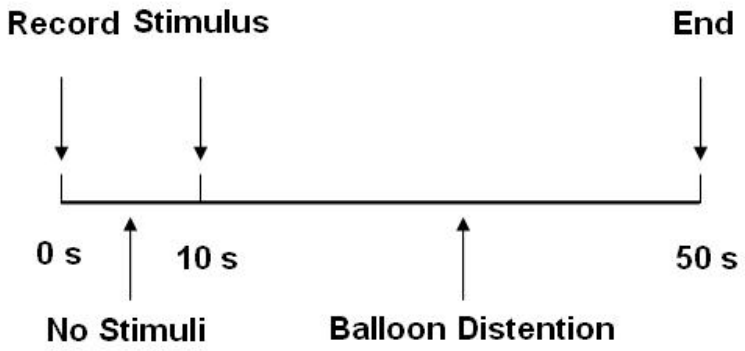
machine. PE-60 tubing was inserted 2-cm into the rectum using lubricant Astroglide (BioFilm, Vista VA) and 100 ul of saline was instilled intracolonically to cleanse the colon. Then, the balloon with a protective sheath was lubricated and was placed into the rectum with the end of the balloon 0.5-cm beyond the anus. The sheath was slowly removed from the balloon and the balloon was taped firmly by autoclave tapes to the tail of the mouse. Next, extra EMG electrodes hidden under the skin of the neck area were carefully removed and the 0.5-cm tip was carefully stripped by forceps. Local anesthetic (lidocaine) was applied to reduce pain due to the incision. Mice were then placed into the restraint tubing. The EMG wire was extracted from the hole in the top of the tube and the opening of the tube was blocked by a 2-cm x 2-cm Gauze pad and tape. The restraint device was covered by a dark fabric with a hole for accessing the EMG electrode to reduce ambient light and ease the habituation of the animal. The EMG electrode was connected to the recording apparatus and the tail was connected to the ground electrode. The CRD balloon was connected to a valve controller (University of Iowa, Iowa city, IO), which was subsequently connected to an oxygen tank (Figure 2.3). The function of the valve controller was to display the gas pressure and automatically control the timing of balloon distention. The gas pressure can be adjusted by the pressure knob in the oxygen tank. Animals were allowed 30-minute to recover from anesthesia and habituate to the device. A 5-minute baseline activity was recorded before the stimulus of distention. Then, the balloon was inflated to 15-, 30-, 45- and 60-mmHg for 40 seconds consequently with three repeats at each level and a 3-minute resting

period between each distention. After distention, the proper position and the patency of the balloon were checked and verified before the animals were euthanized.



(**Figure 2.3 Colorectal distention test.** Upper, Schematic of colorectal distention setup. Balloon and EMG electrode were implanted in an awake mouse that was restricted in a cone. The balloon was inflated by a gas tank and the EMG signal was recorded by CED 1401. Lower, a photo of a mouse in a restraint device during the test. Note that the restraint device was covered by a dark fabric in the test (not shown here). A, EMG electrode connected to neck; B, reference electrode connected to the tail; C, CRD balloon inserted into the rectum and connected to the gas tank.)

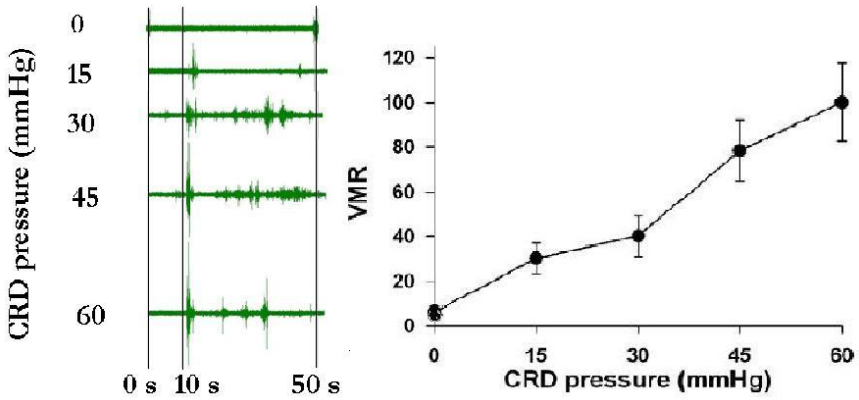
EMG recording. The EMG recording system consistes of an amplifier, an analogue to digital board (Micro-1401, CED, UK) and a computer with Spike2 software (CED, UK). The EMG electrode was connected to the amplifier and the tail was connected to the ground electrode. Baseline activity was recorded for 5 minutes before the stimulus of distention. Then, EMG signals were recorded for 10 seconds before the balloon distention and for 40 seconds during balloon distention (Figure 2.4). If the animal exhibited movement during the 10 second pre-distention period, the distention would be aborted and restarted until the animal became quiescent.



(**Figure 2.4 Experimental paradigm for balloon distention and EMG recording.** The EMG signals in the first 10-second non-stimulation period and in the subsequent 40-second balloon distention period were recorded.**)**

EMG data analysis. The EMG data was counted as the number of spike events above threshold. The representative period of baseline activity and the starting and ending points of each distention were identified by a vertical cursor. The threshold was set by placing a horizontal cursor just above the EMG baseline activity of each animal. The

number of events above the given threshold during the 40-second baseline and each distention was calculated by Spike2 script. The number of spike events in the three repeats at the same pressure was averaged. The final data was plotted as a function of the increase in pressure with standard error bar at each level. In some experiments, the final data was normalized to the percentage of VMR in control animals at the 60-mmHg test. Group differences were evaluated by a linear mixed model. The statistical significance was indicated when the P value was less than 0.05.



(**Figure 2.5 Representative EMG spikes (Left) and analyzed VMR data (Right) to each pressure level of distention.)**

Writhing test. Mice were habituated to a plastic four-liter beaker for 30 minutes. Then, 0.6% acetic acid (1ml/100g) was administrated intraperitoneally. The number of characteristic writhes were counted in 5-minute intervals for a total of 20 minutes.

Drug administration. CTX, DHK, and cephalosporin C were purchased from Sigma

(St. Louis, MO). CTX(20 mg/ml) and DHK (1 mg/ml) were prepared in saline and 10 mg/ml cephalosporin C was suspended in 2% Tween 80 (Fisher, Fair Lawn, NJ). CTX (200 mg/Kg/day) and cephalosporin C (100 mg/Kg/day) were administrated intraperitoneally daily for one week.

Western Blotting. Animals were euthanized and spinal cord tissues were dissected. Tissues were sonicated in PBS buffer containing complete protease inhibitor (Roche), assayed for protein concentration and prepared in SDS loading buffer. 3μg of sample was loaded in 8% SDS polyacrylamide gel and transferred to nitrocellular membrane (Bio-Rad). The membrane was then blocked for 1 h at room temperature in 5% milk PBST blocking buffer and then probed overnight at 4°C with primary antibodies in 1% milk PBST [rabbit anti-GLT-1 antibody, 1:2,000; goat anti-actin antibody, 1:3,000 (Santa Cruz Biotechnology, Santa Cruz, CA)]. After three 10-min washings with PBST, the membrane was incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated goat IgG (1:3,000, Santa Cruz) and rabbit IgG (1:6,000) in 1% milk PBST. The HRP signal was detected by incubation with Enhanced Chemiluminescent Substrate (Pierce) and exposure to X-flim. The further analysis was performed with Image J software on scanned images of the membrane. Actin was used asa loading control.

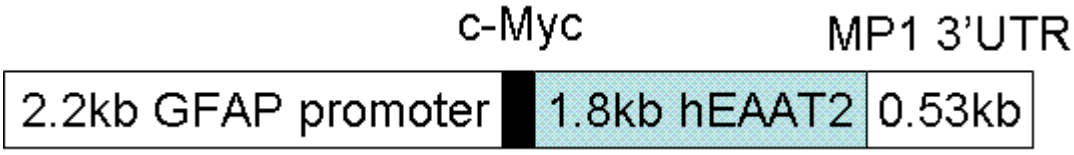
Statistical analysis. The visceromotor response to colorectal distention, performed in

triplicate for each animal, was represented as the mean number of EMG spikes exceeding threshold within the 40-s data analysis window. In some cases, the response was represented as % control, in which the mean response to 60 mmHg CRD in control animals was defined as 100%. In these cases the data were log-transformed, resulting in normally distributed data. For the experiments examining the effect of DHK, group differences were evaluated by a linear mixed model that accounted for the correlation in measures from the same animal and also for the separate variance contributions of replications, pressure levels, and experimental subjects. The linear mixed model is a statistical model containing both dependent variables and independent variables. The dependent variable was the VMR and the independent variables were treatment or genomic group, distension pressure, and the group-by-pressure interaction. The Holm's step-down testing procedure was used to control experimentwise type I error at á=0.05. In the studies involving assessment of VMR to CRD before and after intracolonic ethanol, a paired *t*-test was utilized to assess group differences. Differences in the writhing study were evaluated by Student's *t*-test. In all cases, statistical significance was indicated when $P < 0.05$.

2.4 Results

2.4.1 Visceral nociceptive response was decreased in GLT-1 transgenic mice.

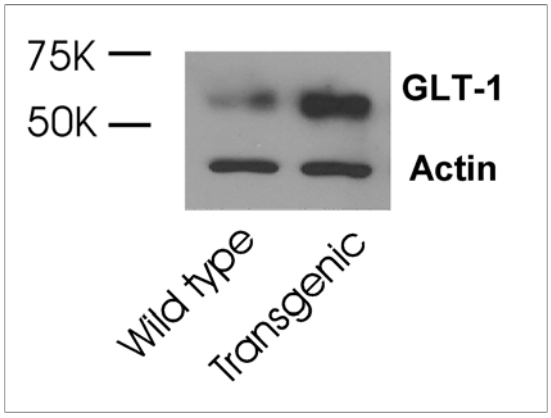
The GFAP promoter drove human GLT-1 transgene expression from prenatal E14.5 to adulthood in astrocytes, where endogenous GLT-1 is normally expressed (Brenner et al., 1994). A c-Myc tag was inserted between GFAP promoter and GLT-1 sequence to detect transgene expression. A segment of Mouse protamine-1 (MP1) gene 3' untranslated region containing polyA-coding sequence was placed in the C-terminal of the construct to enhance transgene expression.



(**Figure 2.6 Transgene construct.** The schematic presentation of the 1.8 kb human GLT-1 cDNA driven by an astrocyte-specific 2.2kb-long glial fibrillary acidic protein (GFAP) promoter with a c-Myc tag in the N-terminal and a mouse protamine-1 (MP1) gene 3' untranslated region in the C-terminal.)

The genotyping based on specific primers in the MP1 and GLT-1 sequence showed a 0.8-Kb GLT-1 transgene band in transgenic animal, while no band showed up in non-transgenic controls. TCR gene was used as PCR control (Figure 2.6). This transgene-specific band revealed that the transgene was integrated into the genomic sequence in the transgenic animal and was passed through generations.

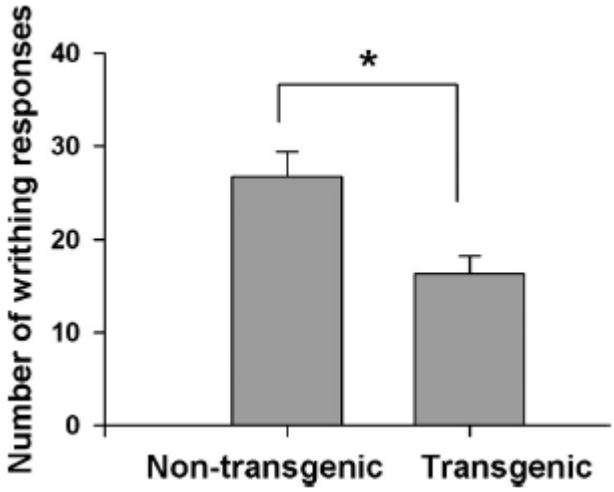
Transgene-coded protein exhibited normal subcellular location and two-fold increase in glutamate reuptake function in spinal cord, compared to wildtype control (Guo et al., 2003; data not shown). The Western blotting showed a 2-2.5-fold increase in spinal GLT-1 protein expression in transgenic mice than wildtype control groups (Fig 2.7). These data were consistent with increased glutamate reuptake activity in the spinal tissue and indicated that the transgene was under successful transcription, translation and trafficking and that transgene-coded protein functioned normally as expected.



(**Figure 2.7 GLT-1 transgene expression.** Representative Western blot of GLT-1 protein levels in the spinal cords of GLT-1 transgenic mice and nontransgenic littermates. In this test, GLT-1 mice exhibited a 2-fold enhanced expression of GLT-1 protein compared with nontransgenic littermates.)

GLT-1 transgenic animals exhibited a 39% reduction in writhing response compared with non-transgenic littermates after intraperitoneal 0.6% acetic acid

injection. Non-transgenic mice (n=35) showed 27±3 writhes in 20 minutes after treatment, while transgenic mice (n=33) exhibited 16±2 writhes during the same period of time (Fig 2.8, $p<0.01$)

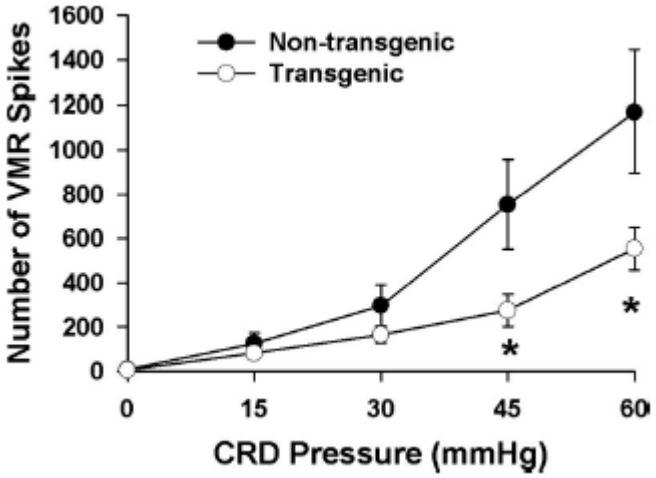


(**Figture 2.8 Writhing response was decreased in GLT-1 transgenic mice.**

Comparison of the writhing response to 0.6% acetic acid in non-transgenic (*n* =35) vs. GLT-1 mice (*n*=33). GLT-1 transgenic mice show 39% less writhing response to acetic acid than non-transgenic littermates. **P* <0.01.)

Because the nociceptive response evoked by intraperitoneal acetic acid involves both somatic and visceral components (Christianson et al., 2007), a viscero-specific method, colorectal balloon distention (CRD), was utilized to measure visceral nociception. The blunted visceral nociceptive response in GLT-1 transgenic mice was confirmed in the CRD model. GLT-1 mice showed a significant reduction in visceromotor response to CRD in assessments of the response to graded distention (Fig

2.9). The visceromotor response was reduced 64% at the 45mmHg distention pressure ($p<0.05$) and by 53% at the 60 mmHg distention pressure ($p<0.05$) in GLT-1 transgenic mice, compared to wildtype littermate controls. There was no significant difference at 15 mmHg and 30 mmHg pressure levels. The CRD data in transgenic animals confirmed that over-expression of glial glutamate transporter GLT-1 attenuated the visceromotor response to noxious colon distention, thus suggesting that a reduction in visceral nociception has occurred.

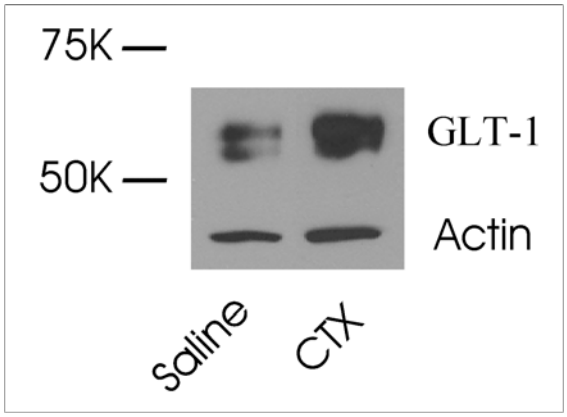


(**Fig. 2.9 VMR response was decreased in GLT-1 transgenic mice.** Comparison of the GLT-1 transgenic and nontransgenic mice visceromotor response (VMR) to graded colorectal distension (CRD). GLT-1 mice (n=12) show a 53–64% reduction in VMR to CRD, compared with non-transgenic littermate controls (n=8). *P <0.05.)

2.4.2 Visceral nociceptive response was decreased in CTX treated mice.

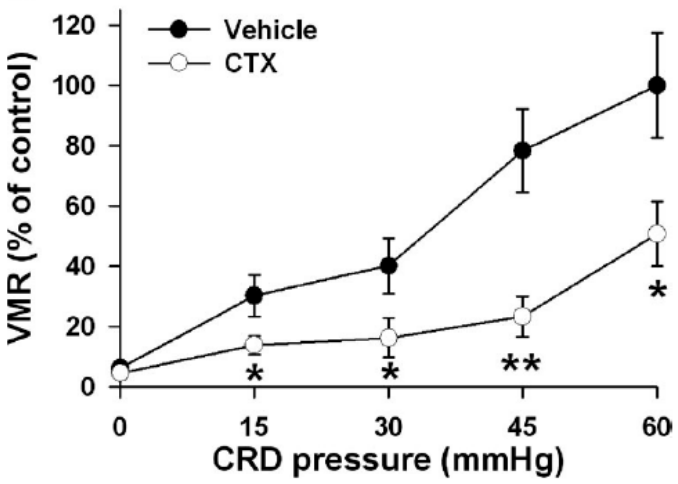
To further assess a possible protective role of enhanced GLT-1 expression in

mediating visceral nociception, a pharmacological approach was utilized to induce GLT-1 over-expression. One week intraperitoneal administration of 200mg/Kg CTX, a beta-lactam antibiotic, enhanced GLT-1 expression at 40~80% in the spinal cord, compared with vehicle-treated control group (Fig 2.10).



(**Fig. 2.10 CTX enhanced GLT-1 expression.** Representative Western blot of GLT-1 protein levels in the spinal cords of CTX- and vehicle (saline)-treated mice. CTX treatment (one week et al., 200mg/Kg, i.p. daily) led to a 44 % increase in GLT-1 protein expression in the spinal cord, compared to saline controlled mice..)

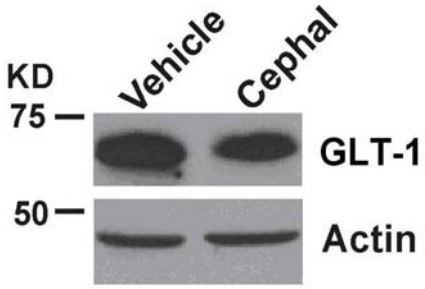
Animals receiving CTX treatment exhibited a 49-70% decrease in the visceromotor response to colorectal distention at each distention pressure, compared to vehicle-treated cohorts (Fig. 2.11), which was consistent with the effect observed in the GLT-1 transgenic mice (Fig. 2.9). These data confirmed that enhancement of GLT-1 expression is associated with a blunted visceral nociception to colorectal distention.



(**Fig. 2.11 CTX treatment decreased VMR to CRD.** The one week CTX-treated mice (*n* =11 et al., 200 mg/ kg/day) showed a 49–70% reduction in VMR, compared with vehicle-treated controls (*n* =10). **P* <0.05; ***P*< 0.005.)

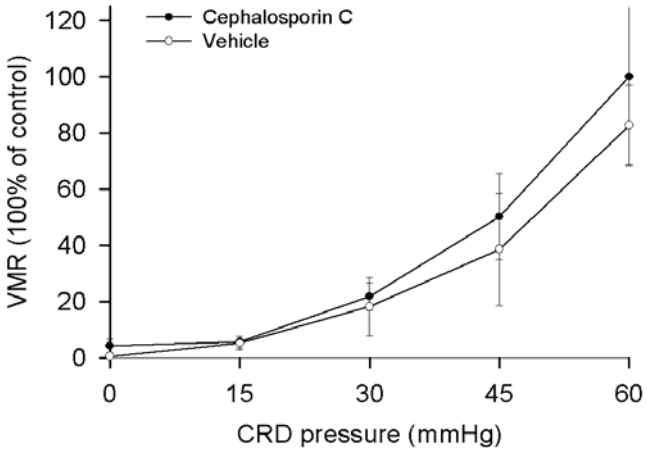
2.4.3 Cephalosporin C treatment did not alter visceral nociceptive response.

To further exclude the possibility of an antibiotic effect of CTX in mediating the blunted visceromotor response to colorectal distention, cephalosporin C, another beta-lactam cephalosporin family antibiotic that does not increase GLT-1 expression (Rothstein et al., 2005), was employed in the colorectal distention model. One week Cephalosporin C treatment at maximal tolerance dose (100mg/Kg) did not alter the GLT-1 expression in the spinal cord, compared to vehicle treated mice (Fig. 2.12, n=3, p>0.05)



(**Fig. 2.12 GLT-1 expression after Cephalosporin C treatment.** Representative Western blot of GLT-1 protein levels in the spinal cords of cephalosporin C- and vehicle-treated mice. One week cephalosporin C treatment (100mg/Kg/day) did not alter GLT-1 protein expression (n=3, $p>0.05$))

One week Cephalosporin C –treated mice showed no significant difference in their visceromotor response to colorectal distention, compared to vehicle control (Fig. 2.13). This study suggests that the protective effect of CTX on visceral nociception was not mediated by antibiotic activity.

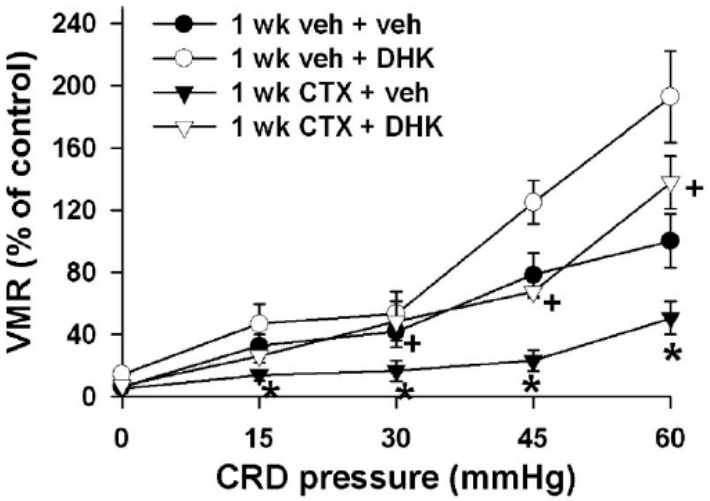


(**Fig. 2.13 VMR to CRD in the Cephalosporin C- and vehicle-treated mice.** One

week cephalosporin C (100mg/Kg) treatment did not alter the visceral nociception in CRD model, compared to vehicle-treated cohorts (n=12, $p>0.05$).)

2.4.4 DHK treatment reversed CTX-blunted visceral nociceptive response.

To further examine whether the reduced visceral nociception was due to enhanced GLT-1-mediated glutamate transport function, DHK, a selective blocker of GLT-1, was intraperitoneally injected into one week CTX-treated mice one hour before the performance of colorectal distention, thereby blocking the GLT-1-mediated glutamate uptake (Rawls et al., 2009). As shown in Fig. 2.14, one hour DHK pretreatment (10 mg/kg) significantly reversed the blunted visceromotor response to colorectal distention produced by one week CTX treatment (n=10, $p<0.05$), whereas DHK pretreatment alone, without CTX treatment, did not significantly affect visceromotor response to colorectal distention (n=10, $p>0.05$). There was a 52–70% decrease in the visceromotor response at all pressures tested in one week CTX-treated mice, compared with vehicle-treated mice (n=10, $p<0.05$). These results suggest that the observed reduced visceral nociception was due to enhanced GLT-1-mediated glutamate transport.



Comparison Groups	Pressure	*P*-value	Symbol
Vehicle vs. CTX	15	0.03	*
	30	0.01	*
	45	0.02	*
	60	0.02	*
CTX vs. CTX+DHK	15	0.12	
	30	0.03	+
	45	0.02	+
	60	0.01	+
Vehicle vs. DHK	15	0.24	
	30	0.23	
	45	0.17	
	60	0.14	

(**Fig. 2.14 Effect of DHK on the CTX-blunted VMR to CRD.** CTX (200 mg/Kg/day) or vehicle was administered intraperitoneally for 7 days in two groups each. One hour before the graded CRD response was elicited, one vehicle and CTX group was treated with either intraperitoneal saline or DHK (10mg/Kg). The one week CTX + ip vehicle (n=10) produced a significantly reduced visceromotor response compared with the one week vehicle + ip vehicle group (n=7) (*P<0.05). DHK significantly reversed the blunted VMR response produced by one week CTX treatment (n=10) (+P<0.05).)

2.5 Discussion

The principal finding of this study was that augmented GLT-1 expression and increased glutamate transport reduce the visceromotor response to CRD. Both transgenic and pharmacological approaches were effective. These findings represent a novel, previously unexplored approach to mitigate visceral nociception.

The effectiveness of systemic DHK to reverse the CTX-blunted VMR provides strong evidence of the involvement of glutamatergic mechanisms in mediating the enhanced GLT-1-induced blunting of the CRD response. Glutamate is a major chemical messenger mediating primary afferent input into the central nervous system. Enhanced GLT-1 glutamate transport potentially leads to reduced extracellular glutamate and reduced activation of glutamate receptors mediating the viscero-nociceptive response. Previous work suggests that activation of lumbar NMDA receptors facilitates visceromotor responses to CRD in the rat (Kolhekar et al., 1994). Thus reduced activation of spinal glutamate receptors is a possible mechanism of the blunted VMR response observed in the present study. Enhanced GLT-1 -mediated glutamate uptake, leading to reduced intrasynaptic glutamate and decreased lumbar glutamate receptor activation, represent testable hypotheses explaining the blunted response of the present study. There may be roles of non-NMDA glutamate receptor mediating the blunted VMR response seen in transgenic and CTX-treated animals. For example, activation of mGlu5 receptors was found to be involved in the transmission of visceral pain in the

spinal cord (Lindström et al., 2008).

Spinal or supraspinal sites of action may be involved in the GLT-1 blunted visceromotor response to CRD. Intrathecal administration of glutamate transport blockers augments nociception; thus it is plausible that enhanced glutamate transporter activity may have antinociceptive effects (Liaw et al., 2005). With regard to putative supraspinal sites of action, descending facilitatory influences affecting visceral nociception from the rostral ventromedial medulla and cortex mediated by glutamate receptor activation has been well described (Urban et al., 1999). The mechanism of GLT-1-blunted visceral pain response awaits further exploration. Reduced activation of spinal glutamate receptors in GLT-1 over-expressing animals after CRD, leading to reduced activation of second order spinal neurons, is a leading possibility. Associated decreases in activated second messenger pathways (PKA, PKC, PIP2, NO/GC/PKC, ERK, p38) downstream from involved glutamate receptors may also be involved. Study of mechanisms of visceral afferent sensitization show that hollow organs are innervated by mechanosensitive receptors that have either low or high thresholds of response, with the latter representing the nociceptor innervations. A putative mechanism of blunted VMR response in GLT-1 over-expressing animals could be a lowered percentage of mechanosensitive high-threshold afferent fibers responding to distension in the noxious range.

Chapter 3

Site of action of GLT-1 over-expression to mitigate visceral nociception

3.1 Abstract

Ceftriaxone (CTX) was recently shown to decrease visceromotor response to colorectal distention and thus potentially mitigate visceral nociception. However, the site of action of this analgesic effect remains unclear. In the present study, I investigated the site of action of GLT-1 upregulation by one-week CTX treatment to mitigate visceral nociception. Here, I report that intrathecal delivery of GLT-1 antagonist DHK to the lumbar spinal cord dose-dependently reversed CTX-blunted visceral nociceptive response, whereas intracisternal delivery of DHK failed to produce visceral anti-nociceptive effects, suggesting a role of spinally located glutamate transporter GLT-1 in visceral nociception. In addition, one-week CTX treatment exhibited no effect on somatic sensory and motor function. These data indicate that GLT-1 upregulation by CTX treatment mitigates visceral nociception through spinal mechanisms.

3.2 Introduction

It has been known for a long time that the spinal cord is important in sensory and nociceptive function. In the early 19th century, the Bell-Magendie law stated that the dorsal portion of the spinal cord was the target of sensory nerve fibers (Bell, 1811). Around 1900s, morphological study revealed that the superficial layer of the spinal cord was specific for nociception that was mediated by un-myelinated C-fiber or thinly-myelinated Aδ-fiber (von Fray, 1895; Cervero et al., 1980). In the 1910s, further investigation showed that the lateral part of the dorsal root, which terminates in the superficial dorsal horn, was responsible for nociceptive reflexes (Sherrington et al., 1906; Ranson et al., 1915).

The current anatomical model of the spinal cord indicates six laminae in the dorsal horn of the spinal cord (Rexed et al., 1952). Lamina Ⅰ is the marginal layer and lamina Ⅱ is known as *substantia gelatinosa.* Lamina Ⅰ and Ⅱ form a clear superficial layer of the spinal cord and receive small diameter fiber input with nociceptive information. Lamina Ⅲ, Ⅳ, Ⅴ and Ⅵ, contain *nucleus proprius* and make up the deep dorsal horn region with less organized neurons and afferent input. Somatic nociceptive information travels along peripheral Aδ and C fibers to spinal laminae Ⅰ, Ⅱ and Ⅲ and synapses on small interneurons, nociceptor specific neurons and wide dynamic range neurons in this region (Cervero et al., 1996). Both of the nociceptor specific neurons and wide dynamic range neurons are also activated by visceral stimuli, such as colorectal diction

(Ness et al., 1991). However, there is not a clear pattern of neuronal organization in the spinal cord. Morphologically similar neurons exhibit distinct physiological functions and diverse types of neurons respond to the same stimuli (Cervero et al., 1996).

There is significant difference between somatic and visceral processing in the spinal cord. First, the primary afferent termination pattern involved in visceral sensory system is distinct from that in somatic sensory system. The visceral C-fibers have both extensive ascending and descending branches in the spinal cord and terminate diversely in lamina Ⅰ, Ⅱ, Ⅳ, Ⅴ, X, and occasionally in the dorsal and lateral funiculi and contralateral lamina V and X (Sugiura et al., 1989). The unique termination pattern of visceral afferents in the spinal cord explains the genesis of referred pain, which is felt in a part of the body at a distance from the area of stimulation (Robinson et al., 2008). The extensive arborization of visceral afferents is also considered as compensation for the relative small number of visceral afferents, which represent less than 7% of the total primary afferents in the DRG (Cervero et al., 1984). Second, visceral afferents exhibit a distinct myelination pattern. For example, 67% of visceral afferents from S1 DRG are unmyelinated C fibers and 33% are Aδ fibers with rare Aβ fibers (Sengupta et al., 1994). In contrast, 70% of afferents from L4 DRG not innervating visceral organs are Aα/β fibers with few Aδ (15%) and C (15%) fibers (Harper et al., 1985). Third, the majority of somatic secondary neurons project axons through the anterolateral system to the supra-spinal region. In contrast, visceral neurons in the spinal cord reply more heavily on the dorsal column pathway, instead of the anterolateral system, to process

nociceptive information (Willis et al., 1999). A lesion of the dorsal column led to a 60-80% decrease in visceromotor response to CRD, whereas a ventrolateral column lesion only reduced visceromotor response by 20% (Willis et al., 1999).

Spinal visceral neurons also receive descending modulation from the supra-spinal region. Thoracic spinal cooling produced differential effects on thoracolumbar spinal neurons that respond to greater splanchnic nerve (Akeyson et al., 1990) and renal nerve stimulation (Standish et al., 1992). It was estimated that the responses of 72% of the neurons were increased, 11% were decreased and 13% were not affected after cooling (Standish et al., 1992). The responses of lumbosacral spinal neurons to noxious colorectal distension also exhibited similar biphasic dose-dependent characters after electrical stimulation of the rostroventral medulla (RVM) (Zhuo et al., 2002). The same effect was also observed following microinjection of glutamate into the RVM nuclei (Zhuo et al., 2002). In contrast, electrical stimulation of the periaqueductal gray (PAG) significantly inhibited the spinal neurons evoked by colorectal distension (Okada et al., 1999).

Spinal neurons exhibit profound changes after chronic persistent pain, as described in chapter 1. Some important molecules, including extracellular signal-regulated kinase (ERK), brain derived neurotrophic factor (BDNF) and adenosine triphosphate (ATP), are upregulated when chronic pain persists, which mediates long term facilitation and central sensitization of spinal neurons.

Our lab previously found that systematic treatment of GLT-1 antagonist DHK

reversed CTX-blunted visceral nociceptive response. But we did not know about the site of action of GLT-1 and whether over-expression of GLT-1 might affect other physiological functions.

In the present study, I found that intrathecal administration of GLT-1 antagonist DHK to the lumbar spinal cord reversed CTX-blunted visceral nociceptive response in a dose dependent manner, whereas intracisternal administration of DHK did not produce anti-nociceptive response. These data indicate that spinal glutamate transporter GLT-1 is functionally important in visceral nociception.

3.3 Methods.

Animals. Two to three-month old FVB/N female mice were used in this study. Mice were maintained on a twelve-hour day- twelve hour-night cycle and were housed in groups of five with water and food. All protocols were approved by the Institutional Animal Care and Use Committee in the Ohio State University and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Drug administration. CTX (Sigma, St. Louis, MO) was prepared in saline (20 mg/ml) and was administrated intraperitoneally (200mg/Kg/day) for one week. Saline was used as control following the same procedure.

Colorectal distention. Colorectal distention (CRD) test was performed as described in chapter 2. Briefly, animals were surgically implanted with two EMG electrodes in the external oblique muscle. Three days after recovery from surgery, the visceromotor response to colorectal distention was elicited by intracolonic balloon distention in triplicate at each of the 15-, 30-, 45- and 60-mmHg level. The data was analyzed as the number of EMG spikes above baseline using Spike2 software (CED, UK).

Intrathecal administration. One hour before colorectal distention, animals were lightly anesthetized by 1.5% isoflurane. The hair in the midline of the back was removed using animal clippers. The location of the lumbar spinal cord was determined by the level of the last rib. A 30-gauge needle connected to a Hamilton syringe was placed vertically at a 90 degree angle through the skin right above the injection site and reached the vertebra. The needle was then lowered to a 15 degree angle to vertebra and was inserted 1mm into the space between the vertebrae. 5µl of DHK at different concentrations (3-, 0.3-, 0.003-mM) was delivered into the spinal cord over one minute. A high concentration of DHK produced slight spontaneous nociceptive behaviors, such as licking and shaking.

Intracisternal administration. One hour before colorectal distention, animals were lightly anesthetized by 1.5% isoflurane. Hair in the dorsal aspect of the neck was

removed by animal clipper. A steel bar with a 0.5-cm diameter was put underneath the neck. The injection site was determined by the obvious gap between the skull and the first vertebra. A Hamilton needle connected to a Hamilton syringe was inserted into the gap and carefully penetrated the dura membrane. 5μl of 0.3-mM DHK was delivered into the cistern magna.

Glutamate uptake assay. The lumbar section of the spinal cord was dissected from animals and minced into small pieces by scissors. Tissue from each mouse was homogenized 10 strokes by a rotor-drived homogenizer in 3-ml ice-clod tissue buffer containing 1X complete proteinase inhibitor, 0.32M sucrose and 0.05M Tris (Ph=7.4). Cell debris was removed by 1,000g centrifugation 10 min at 4℃ and synaptosome in the supernatant was separated into 2 tubes, one with 2ml and the other one with 1ml. Synaptosome was then spinned down at 16,000g 10 min at 4℃. For each animal, uptake was measured in three different groups in triplicate: normal control group, DHK (GLT-1 antagonist) treated group and sodium-free negative group. Synaptosomes were resuspended in 1.5ml Kreb buffer (NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, KH_2pO_4 1 mM, $NaHCO_3$ 25 mM, D-Glucose 0.55 mM, $CaCl_2$ 2 mM, pH=7.4) for first two groups tests, or in 0.75ml Na-free Kreb buffer (Cholone-Cl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, KH_2pO_4 1 mM, Tris-Cl 25 mM, D-Glucose 0.55 mM, $CaCl_2$ 2 mM, pH=7.4) for sodium-free group, respectively. 200μl of synaptosome suspension was loaded into clean eppendorf tubes and was pre-incubated in 37℃ for 10 min. 1mM

DHK was added into the DHK group. To initiate the reaction, 0.1mM unlabeled glutamate and 0.05uM tritium-labeled glutamate (Perkinelmer Inc, USA, 1mCi/ml) were added into each tube and tubes were incubated in 37℃ for 10 min. 500µl 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 4ml PBS buffer and the filter paper was transferred to scintillation vials with 3ml scintillation buffer and 0.1N NaOH. Filter paper received 0.05uM 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 DHK-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.

Rotarod test. Mice were trained on an accelerating rotarod (Columbus Instrument, OH) 3 times per day for 3 days. The cylinder of the rotarod was 71 cm long and its diameter was 3.2 cm. Mice were allowed to explore the rotarod for 2 min without rotation. The velocity of the rod was set to increase from 0 to 40 rpm in 5 min. Mice with stable 300s performance on the 3rd day were used later. After one week 200mg/Kg/day CTX or saline i.p. treatment, mice were tested three times per day for 3 days with 1 hour

interval, following the same protocol. The latency to fall off the apparatus in the three trials was recorded and the 3 day data from individual mouse was averaged. Significant difference was assessed by using Student's t test.

Hot plate test. A 15-cm wide metallic plate surrounded by a transparent box was heated up to 56℃, as measured by a built-in digital thermometer with an accuracy of 0.1^{o}C. Animals were placed on the hot plate and the latency to respond with either a hindpaw lick, hindpaw flick, or jump (whichever comes first) was recorded with a chronometer. Animals were immediately removed from the hot plate and returned to their home cages. If a mouse did not respond within 30 seconds, the test was terminated and the mouse was removed from the hot plate to prevent tissue damage.

Tactile test. Animals were placed in a mesh platform covered with a transparent box and allowed 30 minutes to habituate. The von Fray filaments at 2.44, 2.83, 3.22, 3.61, 4.08 and 4.56-g were used to stimulate both of the hind paws for 3s, starting from 3.61-g filament. Based on the "up and down" method (Chaplan et al., 1994), the positive withdraw response was followed by the application of the next thinner filament, verse vise. Total ten stimuli were applied to one animal and the 50% response threshold was calculated by the formula: 50% g threshold = $(10^{Xf + \kappa\delta})/10\ 000$; where Xf is the value of the last von Frey filament applied; κ is a correction factor based on pattern of responses (from Dixon's calibration table); δ is the mean distance in log units between stimuli

(here, 0.4).

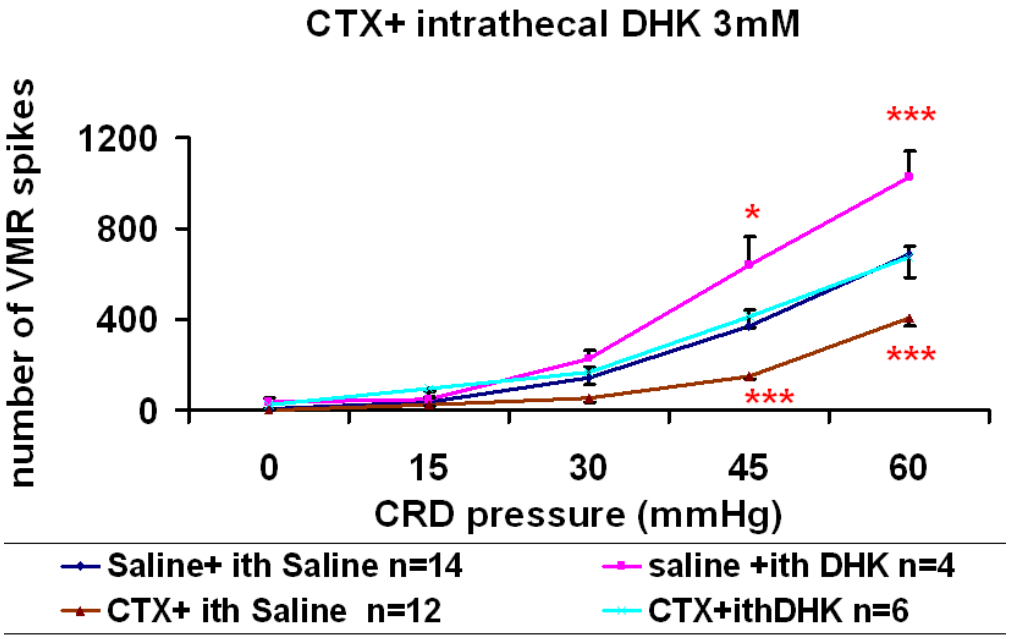
Statistical analysis. The visceromotor response to colorectal distention, performed in triplicate for each animal, was represented as the mean number of EMG spikes exceeding threshold within the 40-s data analysis window. The data was log-transformed, resulting in normally distributed data. In the colorectal distention experiments examining the effect of DHK, group differences were evaluated by one way ANOVA test and Holmes post hoc test. In other experiments, group difference between control and CTX animal were evaluated by Student's *t*-test. In all cases, statistical significance was indicated when $P < 0.05$.

3.4 Results

3.4.1 Intrathecal DHK treatment dose-dependently reversed CTX-blunted visceral nociceptive response.

Spinal cord dorsal horn and brainstem are two major areas regulating visceral nociception (Willis et al., 1999; Ness et al., 1987). To investigate the role of spinal cord GLT-1 in processing distal colon distention-induced visceral pain, 5 μl of 3mM GLT-1 antagonist DHK was delivered to the lumbar section of the spinal cord in the animal model of colorectal distention. Controlled animals received the same amount of intrathecal normal saline injection. One week 200mg/Kg/day CTX treatment produced

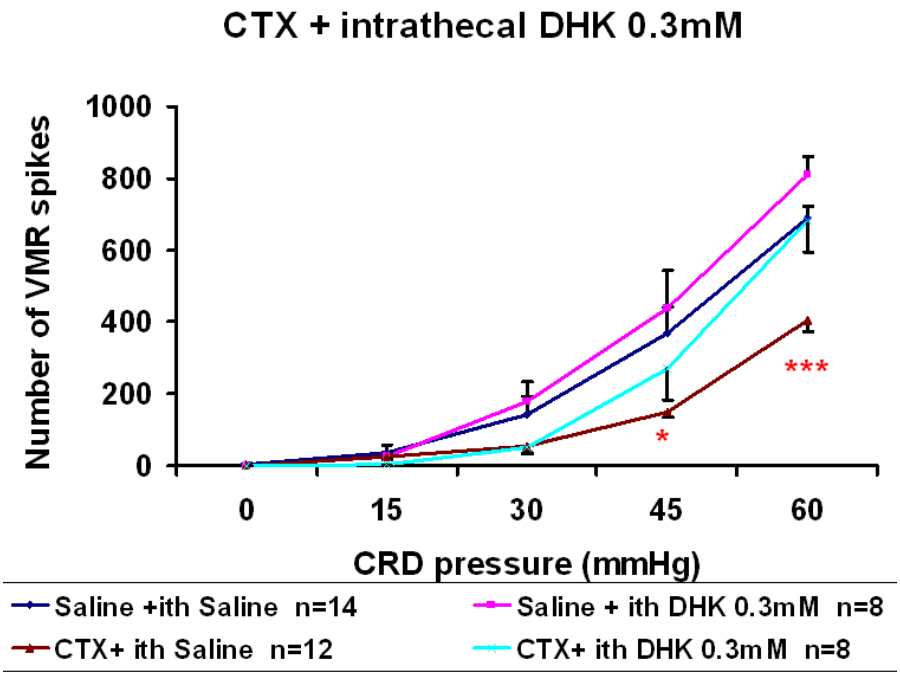
a reduced visceromotor response to 45- and 60- mmHg colorectal distention test ($p<0.005$, n=12). Intrathecal 3mM DHK administration one hour prior to the CRD test reversed the CTX-blunted visceromotor response at 45- and 60- mmHg pressure ($p<0.05$, n=6, Fig 3.1). This suggests the activation of spinal GLT-1 mediates CTX induced hyposensitivity. Of note, intrathecal 3mM DHK administration produced an augmented visceromotor response to 45- and 60- mmHg CRD, compared to intrathecal saline treatment.



(**Figure 3.1 3mM DHK treatment significantly reversed CTX blunted visceromotor response in 45 and 60 mmHg test.** 3mM DHK treatment alone induced visceral hypersensitivity in 45 and 60 mmHg tests. *: $p<0.05$, *** $p<0.005$)

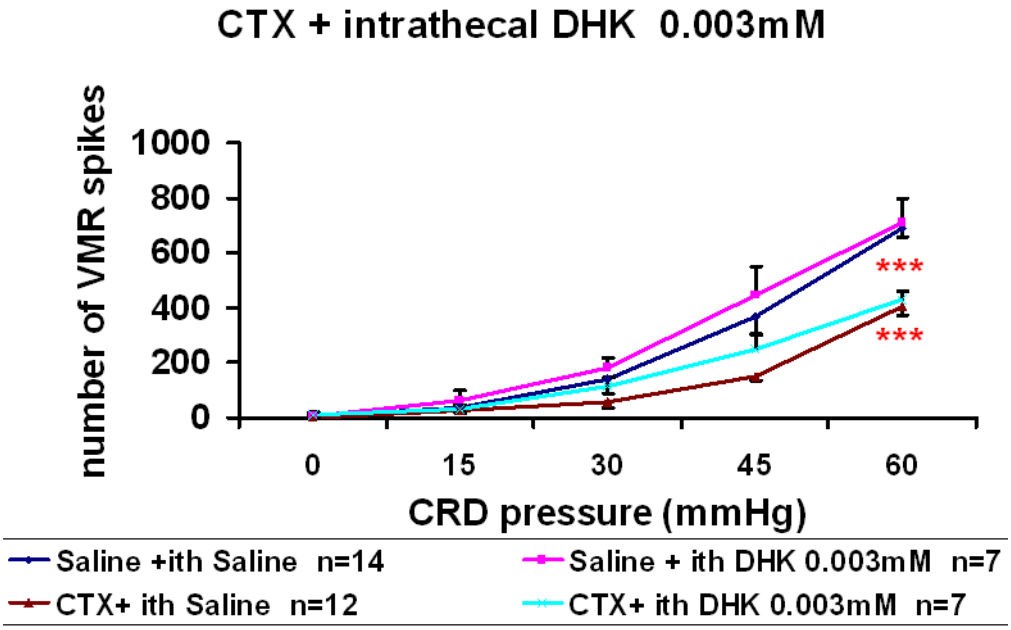
To further investigate the dose-response character of DHK in the spinal cord to reverse

CTX blunted visceromotor response, a ten-fold lower dose of DHK (0.3mM) was delivered intrathecally at the level of the lumbar spinal cord one hour prior to colon distention. Control animals received intrathecal saline. Intrathecal 0.3-mM DHK did not significantly enhance the visceromotor response to 45- and 60- mmHg CRD test (Fig 3.2, $p>0.05$, n=8). When injected into one week CTX treated animals, 0.3-mM DHK was still able to reverse CTX-blunted visceromotor response to 45- and 60-mmHg CRD test (Fig 3.2, $p<0.05$, n=8).



(**Figure 3.2 Intrathecal 0.3mM DHK administration significantly reversed CTX blunted visceromotor response to 45- and 60- mmHg CRD test**. Intrathecal 0.3-mM DHK alone did not produce a response different from intrathecal vehicle injection. *: $p<0.05$, *** $p<0.005$)

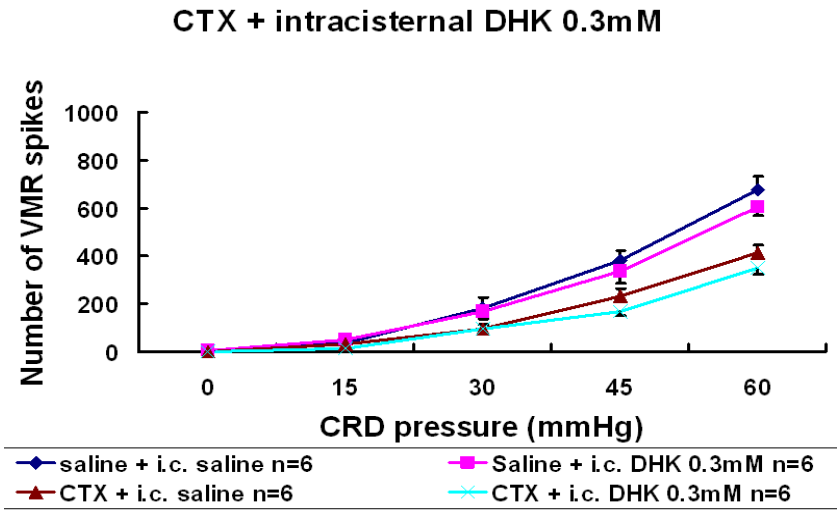
To determine a dose of DHK that does not reverse CTX-blunted visceromotor response, 0.003 mM DHK was administrated intrathecally to the spinal cord. Control animals received the same amount of intrathecal saline treatment in the spinal cord. In the CRD model, intrathecal 0.003-mM DHK did not reverse CTX-blunted visceromotor response to 60- mmHg CRD test, nor affect basal visceromotor response in control animals (Fig 3.3, n=7, $p>0.05$). This experiment suggested 0.003-mM DHK in the spinal cord does not affect visceral sensory function.



(**Figure 3.3 Intrathecal administration of 0.003-mM DHK did not reverse CTX-blunted visceromotor response in CRD model**. *: $p<0.05$, *** $p<0.005$)

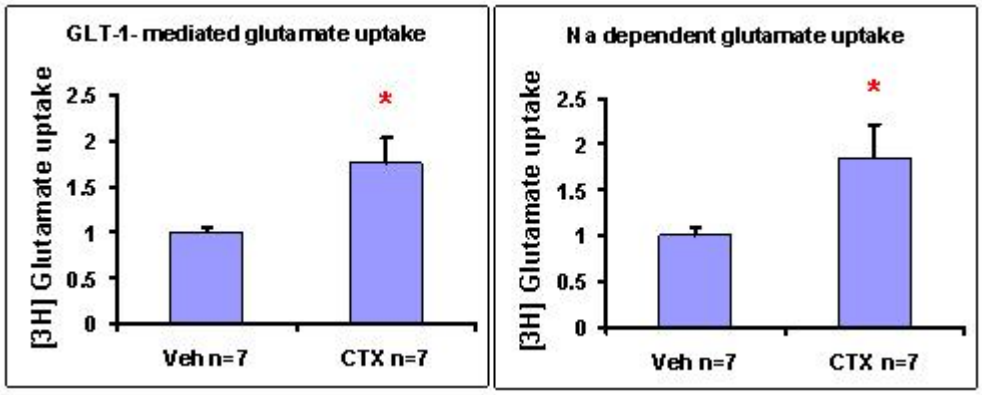
3.4.2 Intracisternal DHK treatment did not reverse CTX-blunted visceral nociceptive response.

To investigate a possible role of the brainstem glutamate transporter GLT-1 in mediating CTX-blunted visceromotor response, DHK was delivered intracisternally one hour prior to CRD at the same dose (0.3mM) found effective in the intrathecal administration. Control animals received an intracisternal vehicle injection. Intracisternal 0.3mM DHK administration did not significantly alter the visceromotor response to colorectal distention in all of the pressures tested, compared to intracisternal saline treated animals (Fig 3.4, n=6, $p>0.05$). In the CTX treated animals exhibiting blunted visceromotor response, there was a lack of effect of intracisternal 0.3mM DHK administration to significantly reverse the CTX-blunted visceromotor response to colorectal distention (Fig 3.4, n=6, $p>0.05$).



(**Figure 3.4 Intracisternal 0.3 mM DHK administration did not alter visceromotor response to colorectal distention**.)

To confirm that one week CTX treatment augments glutamate reuptake activity in the spinal cord, glutamate uptake was investigated in a synaptosome-based tritium-labeled glutamate reuptake assay. GLT-1 antagonist DHK and sodium free buffer system were used to measure GLT-1-mediated glutamate uptake and overall sodium-dependent glutamate uptake. After one week 200mg/Kg/day CTX treatment, there was a 76% increase in GLT-1-mediated glutamate uptake and a 84% increase in sodium-dependent glutamate uptake (Figure 3.5 n=7, $p<0.05$). In conclusion, one week CTX treatment resulted in a 40-80% increase in GLT-1 protein expression (Fig 2.10) and a 76-84% increase in glutamate uptake function (Figure 3.5).

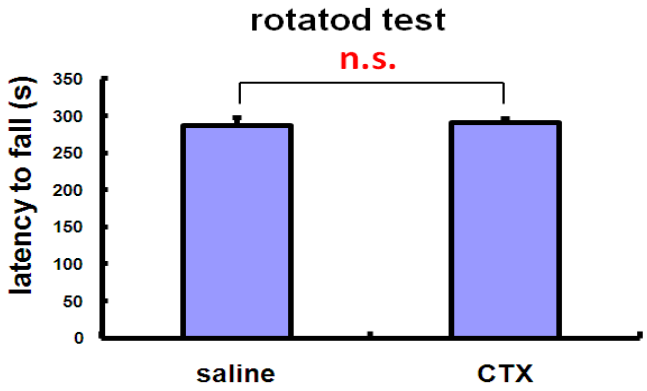


(**Figure 3.5 CTX treatment enhanced glutamate reuptake function.** Left, GLT-1 mediated glutamate uptake. Right, sodium-dependent glutamate uptake. *, $p<0.05$)

3.4.3 CTX treatment did not alter somatic sensory and motor function.

An important question related to possible clinical utility of CTX treatment is to

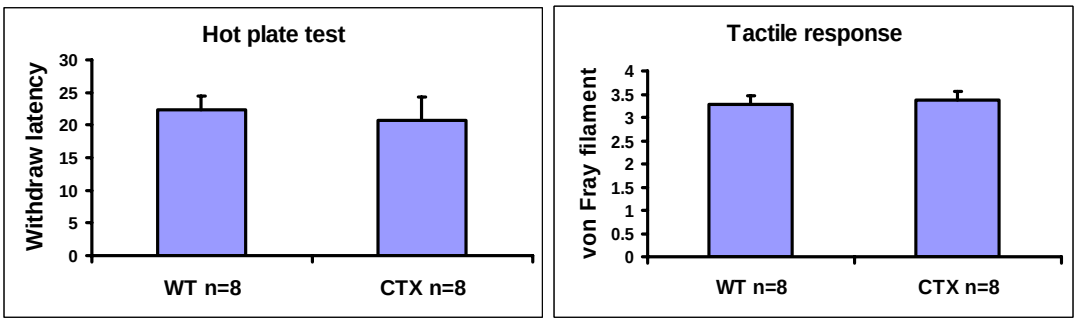
evaluate possible motor function change by GLT-1 over-expression. Rotarod test was employed to evaluate whether GLT-1 up-regulation by one week CTX treatment alters movement coordination and balance. Animals were exposed to the rotarod test after one week 200mg/Kg/day CTX or saline treatment. There was no difference in the falling latency between CTX and saline treated animals (Fig 3.6). These data suggested that GLT-1 upregulation by 200mg/Kg/day CTX treatment did not alter the motor behavioral function in mice and, more importantly, did not affect the motor component of the visceromotor response.



(**Figure 3.6 CTX treatment did not alter motor function**. One week 200mg/Kg/day CTX treatment did not result in a significant change in the falling latency in the rotarod test, compared to control animals.)

To evaluate whether there was a somatic sensory function change due to GLT-1 overexpression, hot plate test and von-Fray tactile test were employed to measure the somatic thermal and mechanical sensory functions, respectively, after one week CTX

treatment. There was no difference in either thermal or mechanical nociceptive test between CTX and saline treated animals (Fig 3.7), suggesting that GLT-1 upregulation by 200mg/Kg/day CTX treatment did not alter somatic sensory function. This result was identical to that observed in the somatic sensory test in EAAT2 transgenic animals (data not shown).



(**Figure 3.7 CTX treatment did not alter somatic sensory function**. Left, the withdraw latency to the 52℃ hot plate test. The mean latencies of one week 200mg/Kg/per CTX treated mice (22.3s, n=8) and vehicle treated mice (20.7s, n=8) were not significant ($p > 0.05$). Right, the mechanical threshold by von Fray filament. The thresholds of CTX treated mice (3.27g, n=8) and vehicle treated mice (3.37g, n=8) were similar ($p > 0.05$).)

3.5 Discussion

The present study demonstrated that spinal GLT-1 overexpression is effective to blunt visceromotor response to colorectal distention, suggesting the spinal cord is an

important site of action for GLT-1 overexpression to mitigate the visceromotor response. In addition, GLT-1 overexpression did not affect somatic sensory and motor function. Because the visceromotor response is composed of visceral sensory and motor components, unaffected motor function by CTX treatment indicates that spinal GL-1 overexpression may attenuate visceral nociception.

Intrathecal delivery of the GLT-1 antagonist DHK at 3-mM concentration significantly reversed the CTX-blunted visceral analgesia effect. This result supports the hypothesis that the spinal glutamate transporter was primarily regulated by CTX and was responsible for the visceral nociception processing. The anti-nociceptive effect of one week CTX treatment has been demonstrated by other labs. For example, intrathecal delivery of CTX produced anti-nociceptive effect to neuropathic pain models in both thermal and mechanical tests (Hu et al., 2009). Moreover, gene transfer of GLT-1 into spinal cord astrocytes by recombinant adenoviruses attenuated inflammatory and neuropathic pain, probably via prevention of central sensitization (Maeda et al., 2008). In addition to CTX, riruzole, another glutamate reuptake activator, was also found to attenuate stress-induced visceral hypersensitivity in rat (Gosselin et al., 2010). Furthermore, visceral normosensitivity relies on the spinal glutamate transporter GLT-1, as intrathecal DHK is sufficient to induce hypersensitivity in normal mice.

The dose-dependent test of DHK revealed that 0.003mM intrathecal DHK did not alter visceromotor response to colorectal distention. These data are consistent with the

IC 50 concentration of DHK (0.045 mM) in vitro, considering the dilution of DHK (0.001mM) in the spinal cerebrospinal fluid (Wang et al., 1998).

The intracisternal injection of 0.3-mM DHK did not significantly alter the visceromotor response to colorectal distention, though the same dose of DHK was effective in the spinal cord site, suggesting that blocking of the brainstem glutamate transporter by intracisternal 0.3 mM DHK might not affect visceral nociception. However, it is important note that in the 60mmHg pressure CRD test, animals receiving intracisternal 0.3mM DHK administration showed a slight trend of decreased visceromotor response, compared to control animals. This experiment does not exclude the role of brainstem glutamate transporter in regulating visceral nociception. First, cistern magna contains larger volume of space, compared to intrathecal space. When the same amount of DHK (5µl) is delivered to both of the two sites, the final DHK concentration would be lower in the cistern magna than that in the intrathecal space. Second, the brainstem consists of many functionally different nuclei that exhibit distinct descending nociceptive regulation. Microinjection of glutamate into the RVM at 5 nmole facilitates responses of spinal neurons to CRD, whereas 50 nmole glutamate inhibits responses of the same neurons (Zhuo et al., 2002). Microinjection of glutamate into the PAG leads to descending inhibition of spinal neurons responding to CRD (Ness et al., 1987). Intracisternal delivery of DHK may affect glutamatergic transmission in one or some of the distinct nuclei in the brainstem. Third, I observed that 0.3mM DHK administration results in a slight trend of decreased visceromotor response to 60 mmHg

CRD stimuli. This effect might be explained by the activation of PAG descending inhibition, because the PAG is located next to the connection between the third and fourth ventricle and DHK was delivered to the forth ventricle. A higher dose of DHK delivery to specific nuclei could be useful to more completely investigate whether GLT-1 mediates an anti-nociceptive effect in the brainstem.

The glutamate reuptake assay showed that CTX treatment promotes significant and similar increases in both GLT-1 mediated glutamate uptake and overall sodium-dependent glutamate uptake. Since sodium-dependent glutamate uptake in the spinal cord is mediated exclusively by GLAST, GLT-1 and EAAC1, These data suggest that GLT-1 protein, instead of GLAST and EAAC1, contributes to the majority of the enhanced glutamate reuptake after CTX treatment, which was consistent with the immunobloting data showing that GLAST and EAAC1 proteins are not altered after CTX treatment (Rothstein et al., 2005). These data also indicate that CTX-upregulated GLT-1 protein displays normal physiological activity, which is consistent with the normal subcellular distribution of GLT-1 protein after CTX treatment (Lee et al., 2008).

There is direct evidence to demonstrate the association of enhanced glutamate reuptake and reduced extracellular glutamate concentration as a result of CTX treatment. Microdialysis data revealed that 200 mg/Kg CTX treatment significantly increased glutamate uptake (24%, $p < 0.001$) and decreased basal glutamate concentration (-59%, $p < 0.001$) in the brain, compared to vehicle treated mice (8.2 uM) (Miller et al., 2008). Moreover, microdialysis data showed that 0.3-mM and 3mM DHK

administration led to 70% and three-fold increase, respectively, in basal extracellular glutamate concentration in the neostriatum area (Fallgren et al., 1996). However, experimental data is still lacking to show how DHK dose-dependently regulate glutamate concentration in the spinal cord. Liaw et al observed that intrathecal 10-mM DHK administration with catheter implantation produced similar spontaneous nociceptive response to the intrathecal administration of 6-mM TBOA, a non-selective glutamate transport inhibitor (Liaw et al., 2005). The following microdialysis data exhibited that 6-mM TBOA led to a significant 39% increase in extracellular glutamate concentration from the baseline of 107 pmol/50 μl (Liaw et al., 2005). Considering microdialysis only measured glutamate spilled from synapse to spinal CSF, it is reasonable to postulate that 3-mM DHK also resulted in a significant increase in the glutamate concentration in the spinal cord synapse.

It is interesting to note that somatic sensory function is not changed by GLT-1 overexpression after CTX treatment, which is confirmed by GLT-1 gene transfer and CTX treatment in the neuropathic pain studies (Maeda et al., 2008; Hu et al., 2010). These data, as well as results from other labs, could be explained by following possibilities that (1) GLT-1 overexpression by CTX treatment or gene transfer might only alter the physiological function of a subset of spinal neurons, which are critical for both neuropathic pain and visceral pain; (2) the spinal neurons responding to neuropathic pain or visceral pain might share some similarities and common pathways, which are different from the spinal neurons responding to somatic acute sensory stimuli.

For example, GLT-1 overexpression by CTX treatment or gene transfer might affect particular subtypes of glutamate receptors in the synapse, which are involved in visceral pain and neuropathic pain, but not in somatic acute pain.

The rotarod test also demonstrated that GLT-1 upregulation by one week 200mg/Kg/day CTX treatment does not alter motor function, which is consistent with the observation that 5 day 200mg/Kg/day CTX treatment did not change performance in the motor function tests, such as plus maze test and open-field test, in normal mice (Miller et al., 2008). These phenomenons might due to following possibilities. (1) Other glutamate transporter subtypes, such as GLAST, might contribute to the motor function process in the central nervous system. For example, the expression of GLAST is much more abundant than GLT-1 in the cerebellum, which controls the motor coordination function (Danbolt, 2001). (2) GLT-1 overexpression by CTX treatment might affect particular subtypes of glutamate receptors, which are involved in sensory function but not in motor function.

Altered glutamatergic neurotransmission in the spinal cord contributes to central sensitization, which mediates hypersensitivity in the neuropathic pain and visceral pain (Latremoliere et al., 2009; Sengupta et al., 2009). Glutamate NMDA receptors are critical for the induction and maintenance of central sensitization (Woolf et al., 1991). Mustard oil instillation was reported to promote cross-organ sensitization and enhance NMDA receptor NR2B subunit phosphorylation and mRNA expression in the spinal cord, suggesting NR2B is involved in visceral hypersensitivity (Peng et al., 2010). Our

unpublished data show that spinal NR2B expression is decreased after GLT-1 upregulation by CTX treatment. Electrophysiologic evidence also reveals that NMDA receptors are vulnerable to the change of glutamate transporters. The blockage of glutamate transport by TBOA leads to the activation of NMDA receptors via glutamate spillover and an increased proportion of NR2B subunit-mediated current (Nie et al., 2009). Intrathecal delivery of the glutamate transport inhibitor TBOA or DHK produces spontaneous nociceptive responses and hypersensitivity that is NMDA receptor-dependent (Liaw et al., 2005). These data are consistent with our findings and suggests GLT-1 upregulation blunts visceromotor response via inactivating NR2B synthesis and phosphorylation. It is also possible to hypothesize that some of the divergent terminations of primary visceral afferents use NMDA receptors for glutamatergic transmission in the deep spinal laminae and dorsal column pathway. This experiment does not exclude the role of other glutamate receptors, such as the AMPA receptor, in visceral nociception. Further experiments are needed to completely investigate the mechanism of the glutamate transporter in visceral nociception processing.

Chapter 4

Colitis-induced visceral hypersensitivity is reversed by CTX treatment

4.1 Abstract

Inflammation of the gastrointestinal tract and accompanied visceral hypersensitivity are commonly found in patients suffering from Crohn's disease and ulcerative colitis. Glial glutamate transporter GLT-1 over-expression by one week CTX treatment was found to mitigate the visceral nociceptive response (Lin et al., 2009). In the present study, the effect of GLT-1 over-expression in an animal model of colitis was investigated. One week CTX treatment upregulated the expression of GLT-1 and reversed TNBS-induced visceral hypersensitivity. In addition, CTX treatment one week after the onset of DSS-induced visceral inflammation could also attenuate the visceral nociceptive response, suggesting a potential therapeutic effect of CTX. MPO assay showed that one week CTX treatment could also reduce inflammation in TNBS-induced colitis; however, one week treatment with cephalothin, another antibiotic that does not induce GLT-1 expression, did not alter TNBS-induced visceral hypersensitivity. These data suggest that GLT-1 over-expression mitigates visceral nociception enhanced by inflammation.

4.2 Introduction

Inflammatory Bowel Disease (IBD) is a type of autoimmune gastrointestinal disorder caused by an abnormal inflammatory response to intestinal microbes in a susceptible host (Abraham et al., 2009). IBD affects 0.5% of the US population and the disease symptoms manifest at 15 to 30 years of age (Loftus et al., 2002). Generally, IBD includes Crohn's disease (CD) and ulcerative colitis (UC). Crohn's disease is a persistent transmural inflammatory bowel disease that might affect any part of the gastrointestinal tract, from mouth to anus .Crohn's disease has a patchy distribution of inflammatory sites in the GI tract, which is transmural through the entire intestinal wall. Some CD patients may develop formation of internal fistulas between the GI tract and other visceral organs, or external fistulas to the skin (Fatahzadeh et al., 2009). In contrast, ulcerative colitis is commonly found in the superficial mucosal layer of the colon and rectum and rarely affects the small intestine. Typical symptoms of IBD include abdominal pain, diarrhea, vomiting and weight loss (Kornbluth et al., 2004).

The etiology of IBD is not clear, but accumulating data suggest that both genetic and environmental factors contribute to the disease onset and progress. For example, smoking is found to be a factor affecting IBD. Smokers are at greater risk for developing Crohn's disease, whereas non-smokers tend to have higher risk of ulcerative colitis (Fatahzadeh et al., 2009). Intestinal bacterial pathogens, defective intestinal epithelial cells and abnormal intestinal immune responses are considered as important

pathological factors contributing to the progression of IBD (Abraham et al., 2009). Antibiotics are effective in decreasing inflammation in some IBD models (Elson et al., 2005). The increased permeability in the paracellular space between epithelial cells and defective tight junction are noted in IBD patients (Turner et al., 2006). Activation and infiltration of innate immune cells are a hallmark of active IBD.

Recent clinical research reveals some important genetic alterations involved in IBD, including the nucleotide oligomerization domain 2 (NOD2) autophagy gene (Abraham et al., 2009). NOD2 is an intracellular protein used to detect bacterial peptidoglycan and is required for the expression of cryptdins, an intestinal anti-microbial peptide. NOD2 knockout mice are susceptible to bacterial infection (Kobayashi et al., 2005). NOD2 mutant mice have elevated NF-kappaB level and cytokine IL-1b secretion, which are associated with increased susceptibility to intestinal inflammation (Maeda et al., 2005).

Besides transgenic models of IBD, chemical inducible IBD models are widely used in basic research, such as the study of visceral pain. Reagents commonly used to induce inflammation in animals include trinitrobenzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS). Mice receiving TNBS enemas develop hapten-induced delay type hypersensitivity and chronic colitis (Jurjus et al., 2004). TNBS is a hapten and it binds to colonic autologous or microbiota proteins as carrier, making them immunogenic to the host immune system. TNBS treatment leads to granuloma with infiltration of inflammatory cells in the intestine, as well as activated macrophages and

lymphocytes with increased inflammatory factors IL-2, IL-12 and IFN-gamma. Thus, TNBS-induced colitis is a Th type-1 response and mimics a Crohn's disease model (Neurath et al., 2005). Inflammation in the colon triggers the release of many inflammatory mediators, including bradykinin, prostaglandin, TNF-alpha and ATP, from mast cells and epithelial cells (Bueno et al., 1999). These chemicals directly stimulate the nerve ending in the mucosa layer of the colon and indirectly trigger the release of other algesic factors such as histamine and nerve growth factor, resulting in visceral hypersensitivity (Bueno et al., 1999). Low dose (0.7mg) TNBS-induced inflammation lasts for 7 days and is diminished after 2 weeks. The increase in colorectal distention-induced visceromotor response in TNBS treated mice also peaks at 7 days and wanes after 2 weeks (Lamb et al., 2006).

Another model of colonic inflammation is the model of DSS. Mice receiving 4% DSS dissolved in drinking water develop ematochezia, body weight loss, mucosal ulcers and infiltration of neutrophils. The susceptibility to DSS and the disease progression varies in different strains of mice (Larsson et al., 2006). The onset of inflammation and bloody stool is at day 5 (C57BL/6), day 6-7 (BALB/c) and day 7 (FVB/N), respectively (Larsson et al., 2006). The inflammation peaks at day 12 and decays thereafter. The visceromotor response to 30mmHg colorectal distention in DSS treated mice is increased at day 5 and reduced in chronic treatment by day 100 (Verma-Gandhu et al., 2007).

DSS directly damages epithelial cells of the basal crypts in the gut and perturbs the

integrity of the mucosal barrier (Wirtz et al., 2007). Chronic colitis by DSS is mediated by activated lymphocytes and CD 4 cells, as well as increased cytokine, beta-endorphin and MOR expression (Okayasu et al., 1990; Verma-Gandhu et al., 2007). Inflammatory mediators, including nitric oxide, bradykinin, prostaglandin, TNF-alpha are released in response to tissue injury and stimulate visceral nerve terminus in the colon.

The myeloperoxidase (MPO) assay is a well-accepted tool to measure tissue inflammatory damage in animal models of colitis. MPO is a peroxidase enzyme abundant in neutrophils and is released upon neutrophil activation by inflammatory pathogens (Allgayer et al., 1991). During inflammation, the synthesis and release of MPO are greatly enhanced. MPO has a unique ability to catalyze chloride and H_2O_2 to form hypochlorous acid, a powerful anti-microbial reagent. This unique property is widely used to detect the amount of MPO in tissue samples.

Many neurochemical systems are altered in animal models of colitis, including adenosine triphosphate (ATP) and glutamate. In the physiological condition, ATP is released from the epithelial cells of viscera and targets P2X-type ATP receptors in the visceral nociceptors (Ferguson et al., 1997). In the condition of visceral inflammation, the release of ATP and the expression of P2X2 and P2X3 receptors are increased in humans (O'Reilly BA et al., 2002; Andersson et al., 2002). The enhanced purinergic activation is correlated with visceral nociceptive stimuli and pelvic nerve excitation in animals (Wynn et al., 2003). P2X3-deficient mice exhibit reduced visceromotor responses to colorectal distention and decreased hypersensitivity to zymosan instillation,

compared to wildtype mice (Shinoda et al., 2008).

In addition to the peripheral ATP system, it is established that spinal glutamatergic neurotransmission plays an important role in visceral hypersensitivity and central sensitization. Visceral tissue injury or inflammation induces an increase in firing frequency of the visceral afferent and leads to central sensitization, which in turn, contributes to chronic visceral pain (Urban et al., 1999; Zhou et al., 2009). NMDA glutamate receptors are well known to mediate central sensitization in the spinal cord (Woolf et al., 1991). Rodent study of colitis induced by intracolonic TNBS demonstrates that the NMDA-mediated current is increased three-fold after the establishment of colitis, which is accompanied with a 28% increase in NR2B expression in DRG neurons and a selective increase in NR1 alternative splicing in the spinal cord (Zhou et al., 2009; Li et al., 2006). The activation of NMDA receptors is partially mediated by phosphorylation of the NR2B subunit via the Src family of tyrosine kinase and ephrinB2 signaling (Slack et al., 2008; Peng et al., 2010). Consistent with the activation of NMDA receptors, two markers of central sensitization, c-Fos and phospho-ERK, are also increased in the spinal cord after colon inflammation (Eijkelkamp et al., 2007; Qiao et al., 2008). These data suggest that spinal NMDA-type glutamate receptors, at least partially, account for the change in visceral nociceptive function in the central nervous system. In addition, TNBS also induces upregulation of brain derived neurotrophic factor (BDNF), calcitonin gene related peptide (CGRP) and phospho-Akt in the spinal cord (Qiao et al., 2008; Qiao et al., 2009).

However, much less is known about the role of glutamate transporters in inflammation-mediated visceral hypersensitivity. The present study investigated the effect of over-expression of the physiologically dominant glutamate transporter GLT-1 in colitis-induced visceral hypersensitivity. Both pre-emptive and therapeutic upregulation of the glutamate transporter GLT-1by pharmacological approach reduced visceral nociceptive response in the animal models of colitis, suggesting GLT-1 upregulation may provide a therapeutic approach to mitigate visceral nociception caused by inflammation

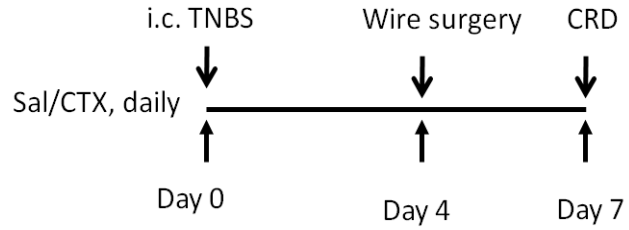
4.3 Methods

Animals. Young adult FVB/N mice aged two to three months were used in this study. Mice were housed in groups of five with twelve-hour day- twelve-hour night cycle and free access to water and food. All protocols were approved by the Institutional Animal Care and Use Committee in the Ohio State University and adhered to the guideline of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Drug administration. Ceftriaxone (CTX), cephalothin (CLT), trinitrobenzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS) were purchased from Sigma (St. Louis, MO). Ceftriaxone and cephalothin (20 mg/ml) were prepared in saline and TNBS (7

mg/ml) was prepared in 50% ethanol. 4% DSS was dissolved in ddH2O as drinking water to animals and each mouse was supplied with 5 ml of 4% DSS everyday. Ceftriaxone and cephalothin (200mg/Kg) was administrated intraperitoneally daily at 10AM for one week. TNBS (7mg/ml, 0.1 ml) was delivered intracolonically through a syringe attached to a lubricated polyethylene catheter (PE20) 2cm proximal to the anus. After instillation, mice were held head-down by lifting up the tail for 1 min to ensure exposure of distal colon to TNBS. Mice were utilized 7 days after TNBS instillation for behavioral tests, MPO assay, western blot and histology analysis (Fig 4.1A,C). FVB/N mice receiving 4% DSS began to develop bloody stools on day 7. From day 8 to day 14, mice were switched to normal drinking water and received one week CTX treatment (Fig 4.1B). Control animals received normal drinking water and saline administration. Mice were harvested at day 14 for further study.

A



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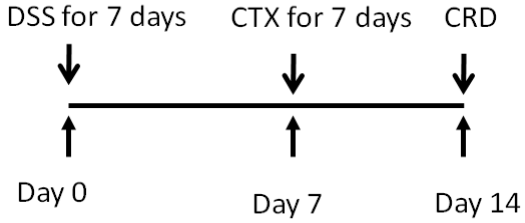

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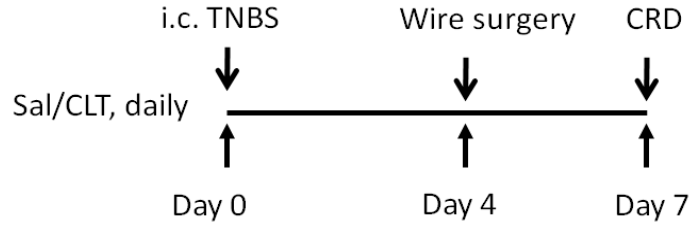

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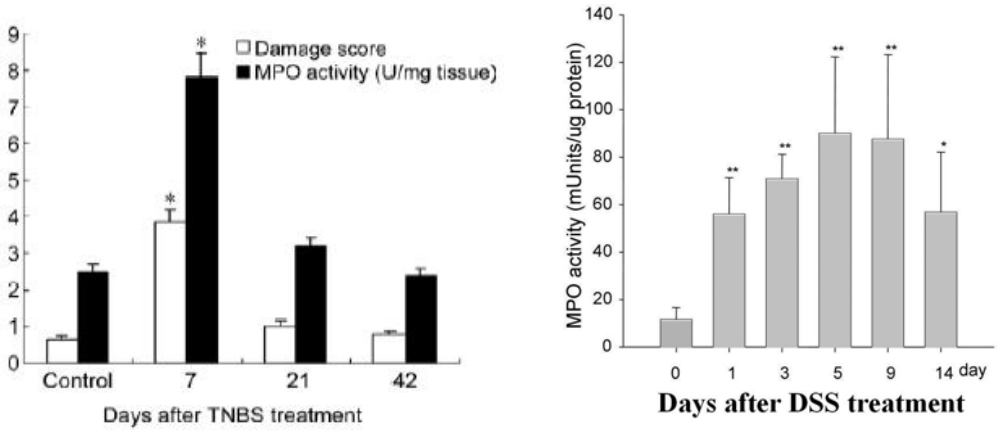


(**Figure 4.1 Schematic for colitis induction, treatment and visceral nociception test.**

(A). Animals received 0.7mg TNBS instillation and began one week 200mg/Kg/day

CTX treatment at day 0. After 3 days recovery from wire surgery, mice received

colorectal distention test on day 7. (B). Animals began one week 4% DSS treatment at day 0. After the establishment of colitis, mice began one week 200mg/Kg/day CTX treatment at day 7. After 3 days recovery from wire surgery, mice received colorectal distention test at day 14. (C). Animals received 0.7mg TNBS instillation and began one week 200mg/Kg/day CLT treatment at day 0. After 3 days recovery from wire surgery, mice received colorectal distention test at day 7. (D). Time course of MPO activity after TNBS (left) and DSS (right) treatment (Adapted from Yan et al., 2009 and Yang et al., 2008).)

Western Blotting. Western blotting was performed as described in chapter 2. Briefly et al., 20 ug of protein sample dissected from mice lumbosacral spinal cord was sonicated, loaded in 8% SDS-PAGE gel and transferred to nitrocellular membranes. After one hour blocking, the membranes underwent over-night primary antibody (rabbit anti-GLT-1 antibody, 1:1000; rabbit anti-actin antibody, 1:1000, Santa Cruz) and one hour secondary antibody (HRP-conjugated rabbit IgG, 1:1000, Santa Cruz) incubation, followed by X-film exposure with Enhanced Chemiluminescent Substrate (Pierce).

Colorectal distention. Colorectal distention (CRD) test was performed as described in chapter 2. Briefly, animal received abdominal surgery to implant two EMG electrodes in the external oblique muscle. Three days after recovery from surgery, the visceromotor response was measured by intracolonic balloon distention at graded 15-

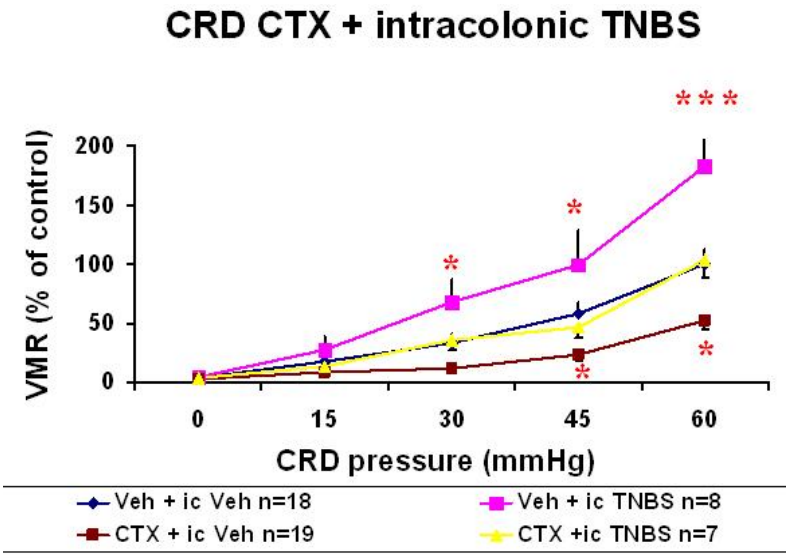
30-45-60 mmHg distention pressures. The data was analyzed as the number of EMG spikes above baseline using Spike2 software (CED, UK) and were normalized to the response in control animals at the 60 mmHg level.

MPO assay. 1 cm of the distal colon was dissected from animals. Samples were minced and homogenized 30 strokes by a motor-driven grinder in 1 ml ice-cold 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma). Samples subsequently underwent 30s sonication and three freeze-thaw cycles in liquid nitrogen. After brief sonication, samples were centrifuged at 10,000rpm for 10 min at 4℃. Supernatant was collected to measure MPO activity. 200ul of 50mM potassium phosphate (pH 6.0) containing 0.167 mg/ml O-dianisidine dihydrochloride (ODD, Sigma) and 0.0005% hydrogen peroxide was loaded as reaction buffer in a 96 well plate. 20ul of supernatant sample was then mixed with reaction buffer. Two minutes later, absorbance was read at 460nm by a spectrometer (PowerWave XS, BioTek). Protein concentration was accessed by adding the 1ul sample into 200ul coomassie blue buffer (Pierce) in the same 96 well plate and absorbance was read at 595nm. The unit of MPO was defined by the change in 460nm absorbance per mg of protein sample in two minutes.

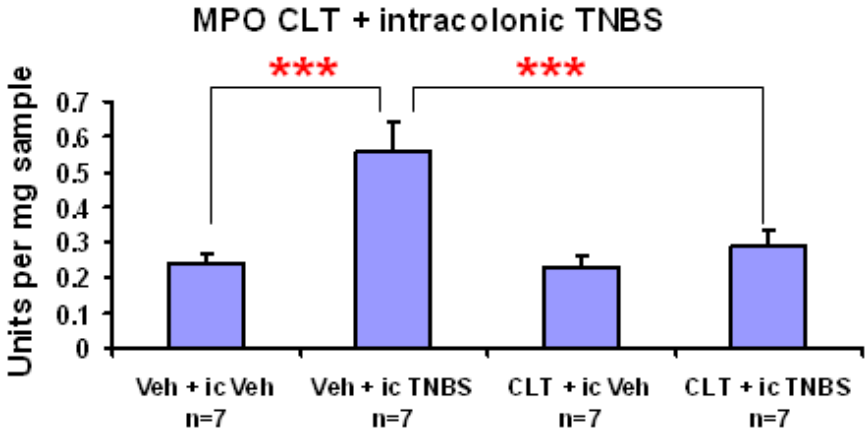
4.4 Results

4.4.1 CTX treatment reversed visceral hypersensitivity induced by TNBS.

In the TNBS induced colitis model, 0.1 ml of 7 mg/ml TNBS was instilled into the distal colon at day 0 and 200 mg/Kg CTX was administrated intraperitoneally daily from day 0 to day 6. Mice were harvested or received CRD test at day 7 (Fig 4.1A). The one time instillation of TNBS resulted in a significant increase in the visceromotor response to the 30-, 45- and 60- mmHg CRD, compared to control animals (Fig 4.2, $n=8$, $p < 0.05$ in 30- and 45- mmHg level, $p < 0.005$ in 60- mmHg level). One week CTX treatment after TNBS instillation reversed colitis-enhanced visceromotor response to the 30-, 45- and 60- mmHg CRD, compared to TNBS treated animals (Fig 4.2, $n=7$, $p < 0.05$). To investigate whether CTX treatment affects colonic inflammation accompanied with the altered nociceptive response, MPO activity was measured in distal colon samples. TNBS treatment led to a significant increase in MPO activity, compared to vehicle treated animals (Fig 4.3, $n= 9$, $p < 0.005$). One week CTX treatment alone did not significantly affect MPO activity in the colon, compared to vehicle treated animals (Fig 4.3 $n=10$, $p > 0.05$). However, CTX treatment inhibited colonic inflammation after TNBS treatment (Fig 4.3, $n=9$, $p < 0.005$). These data suggested that CTX treatment and resultant upregulation of GLT-1 were able to attenuate the TNBS-elevated visceromotor response, as well as colitis.



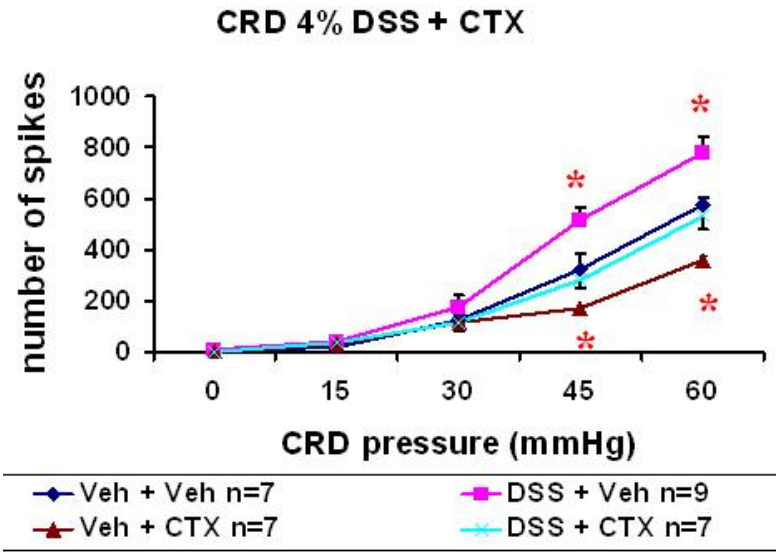
(**Figure 4.2 CTX treatment reversed visceral hypersensitivity induced by TNBS**. One week CTX treatment (200mg/Kg/day) in TNBS treated mice reversed the colitis-elevated visceromotor response in the 30-, 45- and 60- mmHg CRD tests, compared to TNBS treated animals. *, $p < 0.05$; ***, $p < 0.005$)



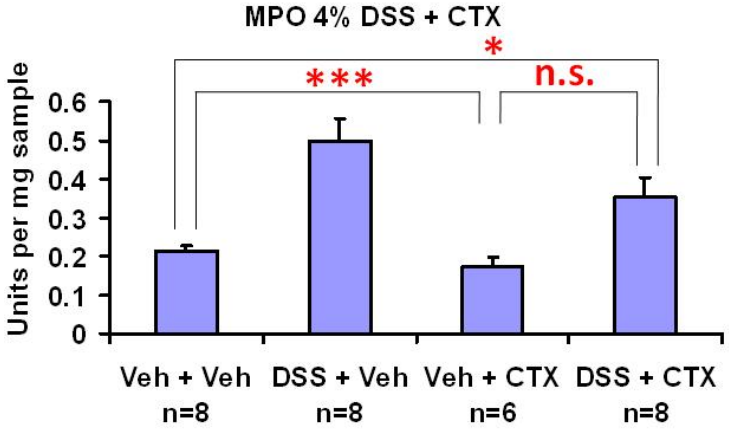
(**Figure 4.3 Colonic inflammation after TNBS and CTX treatment**. One week g CTX treatment (200mg/Kg/day) attenuated colonic inflammation caused by intracolonic TNBS instillation, as measured by MPO activity. One week CTX treatment alone did not alter baseline MPO level in the colon. ***, $p < 0.005$)

4.4.2 CTX treatment reversed visceral hypersensitivity induced by DSS

In the DSS-induced colitis model, 5ml of 4% DSS drinking water was provided to mice everyday for one week. 200 mg/Kg CTX was administrated intraperitoneally daily from day 7 to day 14. Mice were harvested or received the CRD test at day 14 (Fig 4.1B). After one week DSS treatment, animals exhibited bloody stool and decreased body weight at day 7. Control animals receiving normal drinking water did not exhibit any abnormal symptoms. At day 14, DSS treated animals displayed a significantly increased visceromotor response to the 45- and 60- mmHg CRD, compared to control animals (Fig 4.4 n=9, $p < 0.05$). At day 14, DSS treated animals also exhibited severe colitis in the distal colon, as measured by a significant increase in MPO activity compared to control animals (Fig 4.5, n= 8, $p < 0.005$). After the establishment of colitis, one week 200mg/Kg CTX treatment reversed the DSS-enhanced visceromotor response to the 45- and 60- mmHg CRD tests, compared to DSS treated animals (Fig 4.4, n=7, $p < 0.05$). However, CTX treatment from day 7 to day 14after the establishment of colitis produced only a non-significant trend to reduce colonic inflammation caused by DSS treatment (Fig 4.5, n=8, $p > 0.05$). CTX treatment alone also did not significantly alter baseline MPO activity in the colon, compared to vehicle treated animals (Fig 4.5 n=8, $p > 0.05$). These data suggest CTX treatment and resultant upregulation of GLT-1 inhibited the DSS-enhanced visceromotor response without significantly reducing inflammation.



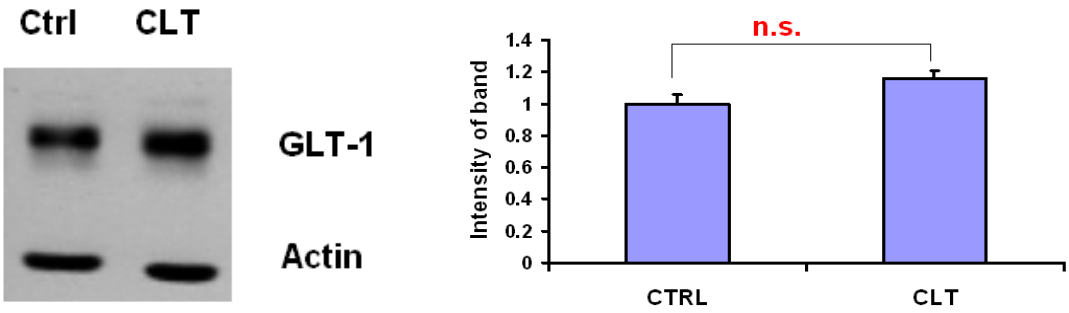
(**Figure 4.4 CTX treatment reversed visceral hypersensitivity induced by DSS**. One week CTX (200mg/Kg/day) treatment after the establishment of colitis reduced the visceromotor response to the 30-, 45- and 60- mmHg CRD tests, compared to DSS treated animals. *, $p < 0.05$)



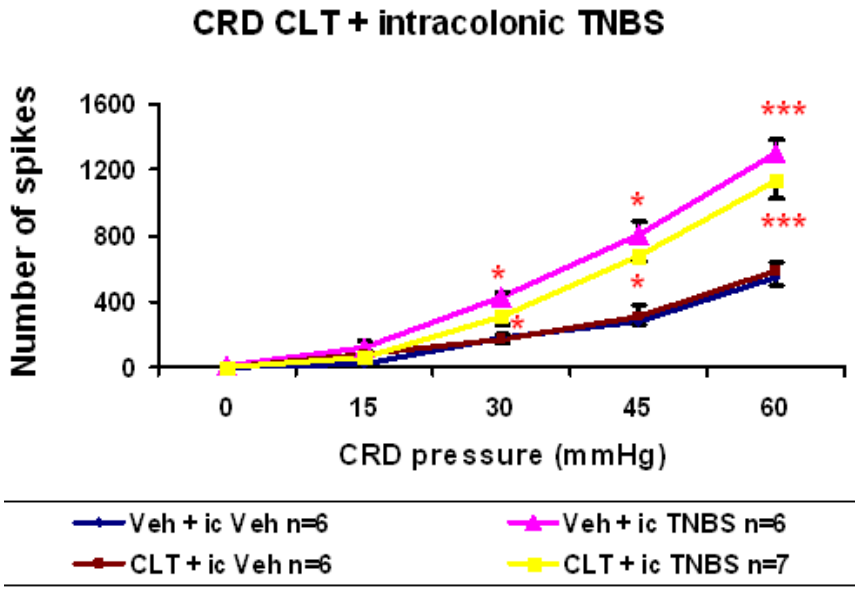
(**Figure 4.5 Colonic inflammation after DSS and CTX treatment**. One week CTX treatment did not significantly attenuate the enhanced MPO activity caused by DSS treatment. CTX treatment alone did not alter the baseline MPO level in the colon. ***, $p < 0.005$; *, $p < 0.05$; n.s., no significant.)

4.4.3 Cephalothin treatment did not reverse visceral hypersensitivity induced by TNBS

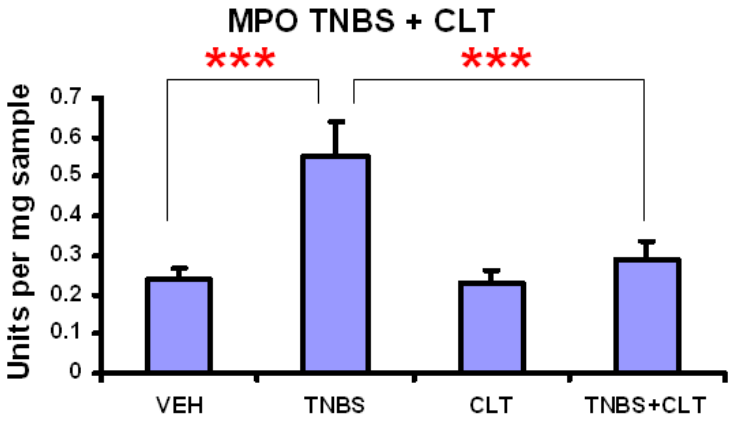
To investigate whether the anti-inflammation effect of CTX contributes to its anti-nociceptive effect on colitis, cephalothin (CLT), another cephalosporin antibiotic, was utilized to investigate its effect on the visceromotor response in colitis induced by intracolonic TNBS instillation. Immunoblotting data showed that one week administration of CLT (200mg/Kg/day) did not increase the GLT-1 protein level in the spinal cord, compared to vehicle treated mice (Fig 4.6). In addition, one week CLT treatment did not alter the basal visceral nociceptive response in the CRD test, compared to vehicle treated mice (Fig 4.7). As described previously, TNBS treatment resulted in a significant increase in the visceromotor response to the 45- and 60- mmHg CRD tests (Fig 4.7), as well as a robust increase in MPO activity in the colon (Fig 4.8). Mice receiving one week CLT + TNBS treatment also exhibited similar increased visceral hypersensitivity to the 45- and 60- mmHg CRD tests compared to vehicle treated mice (Fig 4.7). Thus CLT was ineffective in reducing the TNBS-induced visceral nociceptive response. However, one week CLT treatment reduced the inflammation caused by TNBS without reducing the TNBS-enhance nociceptive response (Fig 4.8). These data demonstrated that the anti-inflammatory property of antibiotics could not alone produce anti-nociceptive effects, suggesting that the apparent analgesic effect of CTX in the colitis model is not mediated solely by an anti-inflammatory effect and that its GLT-1 upregulation properties are implicated.



(**Figure 4.6 CLT treatment did not alter spinal GLT-1 expression**. Left, Representative Western blot of GLT-1 protein levels in the spinal cords of CLT- and vehicle- treated mice. Right, intensity analysis of western bands showed one week CLT treatment (200mg/Kg/day) did not alter GLT-1 protein expression (n=3, p>0.05))



(**Figure 4.7 CLT treatment did not reverse visceral hypersensitivity induced by TNBS**. One week 200mg/Kg CLT treatment after intracolonic TNBS instillation did not alter the colitis-elevated visceromotor response in the CRD test, compared to one week Vehicle + TNBS treated animals. CLT treatment alone did not alter the visceromotor response in the CRD test. *, $p < 0.05$; ***, $p < 0.005$)



(**Figure 4.8 CLT treatment inhibited the enhanced MPO activity caused by TNBS**. One week CLT treatment (200mg/Kg/day) attenuated colonic inflammation caused by intracolonic TNBS instillation, as measured by MPO activity. CLT treatment alone did not alter the baseline MPO level in the colon. ***, $p < 0.005$)

4.5 Discussion

In the present study, GLT-1 upregulation by one week CTX treatment effectively reduced visceral hypersensitivity in animal models of colitis. In addition, one week CTX treatment produced a significant anti-inflammatory effect in animals with intracolonic TNBS treatment.

In both of the TNBS- and DSS- induced colitis models, GLT-1 upregulation by one week CTX treatment was sufficient to mitigate the visceromotor response to colorectal distention (Fig 4.2 and 4.4). These data are consistent with previous work showing that GLT-1 upregulation reduced the nociceptive response to colon distention (Lin et al., 2009).

In addition to the anti-nociceptive effect mediated by GLT-1 upregulation in the spinal cord described in chapter 3, one week CTX administration also exhibits an anti-inflammatory effect in TNBS-induced colitis (Fig 4.3). This anti-inflammatory effect may account in part for the reduced nociception, because inflammation could facilitate the release of ATP and/or histamine, stimulate their receptors in nociceptors and activate silent nociceptors (Bueno et al., 1999).

In the DSS-induced colitis model, GLT-1 upregulation by one week CTX treatment was also able to mitigate the visceromotor response to colorectal distention when administrated after the establishment of colitis (Fig 4.4). However, enhanced colon inflammation induced by DSS was not significantly reduced by one week CTX treatment after establishment of colitis (Fig 4.5). Thus, these data suggest that one week CTX treatment exhibits an anti-nociceptive effect primarily by enhancing spinal glutamate transporter expression. Several reasons may be accounted for the lack of effect of one week CTX treatment to significantly reverse DSS- elevated inflammation. First, unlike TNBS as a hapten, DSS is able to directly destroy the colonic epithelial layer and results in tissue inflammation, whereas gut flora are not a necessary factor for inflammation induction and maintenance (Jurjus et al., 2004). Second, in the DSS + CTX model, CTX is delivered after establishment of colitis. It is possible that one week CTX produces an anti-inflammatory effect better in the early induction stage of colitis (day 1-6) than in the late maintenance stage (day 7-14).

CLT, a cephalosporin antibiotic that did not regulate GLT-1 expression, resulted in

decreased MPO activity but failed to reverse TNBS induced visceral hypersensitivity (Fig 4.7 and 4.8). In addition, the anti-nociceptive effect of one week CTX is dose dependently reversed by the selective GLT-1 antagonist DHK (Fig 3.1, 3.2 and 3.3). Thus, the data taken together suggests that the anti-nociceptive effect of one week CTX treatment could be independent of its anti-inflammatory effect, and confirms the importance of GLT-1 upregulation in reducing visceral nociception.

Chapter 5

Conclusions and perspectives

This dissertation aims to examine the role of the glial glutamate transporter GLT-1 in visceral nociception. Irritable Bowel Syndrome is a common disease in the US with severe abdominal pain. Upregulation of GLT-1 was discovered after one week CTX treatment (Rothstein et al., 2005) and was reported to attenuate neuropathic pain (Hu et al., 2009). In this dissertation, visceral nociception was approached by abdominal EMG recording in colorectal distention model in mice. Nociception tests in the transgenic mice overexpressing human GLT-1 protein revealed that visceral nociception, but not somatic nociception, was decreased by upregulation of GLT-1. This phenomenon was later confirmed in the animals receiving CTX treatment to enhance GLT-1 expression and the animals receiving selective GLT-1 antagonist DHK treatment.

The transgene model and CTX treatment lead to the induction of GLT-1 in the entire central nervous system. There are several important structures in the CNS that could regulate the nociception process. Where is the site of effect of GLT-1 overexpression to mitigate visceral nociception? Based on the nociceptive response observed in intrathecal and intracisternal delivery of DHK, GLT-1 in the lumbar spinal cord may be involved in regulating visceral nociception. This spinal GLT-1-mediated

visceral nociceptive regulation may involve the change of glutamatergic transmission.

Inflammatory Bowel Disease is another disease associated with visceral pain. Inflammation in the colon could result in a change in glutamatergic transmission in the spinal cord (Zhou et al., 2009; Peng et al., 2010). In this dissertation research, GLT-1 upregulation by one week CTX treatment reversed visceral hypersensitivity in animal models of colitis. This anti-nociceptive effect may be independent of the anti-inflammatory effect and relied on spinal cord GLT-1 induction, since another antibiotic CLT decreased inflammation but did not attenuate visceral hypersensitivity.

The mechanism of visceral pain is not completely understood and few therapies are effective to relieve visceral pain. Glutamate transporters may be important in regulating visceral nociception. This dissertation provides novel insight into the treatment of visceral pain.

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