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[ChTitle] Spinal GABA mechanism in neuropathic pain after spinal cord injury

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Abstract:

Spinal cord injury (SCI) often cause sensitization of spinal dorsal horn excitatory neurons via disruption of inhibitory outputs that results in exaggerated nociceptive transmission. Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter and thought to be critical for spinal inhibitory synaptic transmission. However, SCI causes hypofunctional GABAergic inhibitory outputs via multiple mechanisms including loss of GABAergic neurons, downregulation of GABA synthesis enzyme, decrease of primary afferent innervation into GABAergic neurons, and shift of Cl^- gradient in the spinal dorsal horn. These disruptions of GABAergic inhibitory outputs critically contribute to neuronal hyperexcitability in the spinal dorsal horn and chronic neuropathic pain states following SCI. In this book chapter, we focused on spinal GABAergic mechanisms on chronic neuropathic pain development following SCI in rodent animals.

Key Words: Dorsal horn neurons. GABA, Hyperexcitability, Neuropathic pain, Spinal cord injury

1. Introduction

The overall prevalence of neuropathic pain is estimated at 60% - 80% among spinal cord injury (SCI) patients (Gruener et al., 2020; van Gorp et al., 2015). Following SCI, the spatial and temporal changes, as a result of the pathophysiology changes, in the injured spinal dorsal horn lead to newly developed nociceptive synaptic transmission, which is thought to be responsible for the development/maintenance of chronic neuropathic pain (Ferguson et al., 2012; Kang et al., 2020; Lee-Kubli et al., 2016). Under physiological conditions, the sensory synaptic transmission is well regulated via the balance between excitatory and inhibitory circuitries in the spinal dorsal horn. However, imbalance of this regulation led by long-lasting neurochemical and neuroanatomical plasticity changes causes the development of new nociceptive synaptic-circuits that result in hyperexcitability of spinal dorsal horn neurons (Berrocal et al., 2014; Gruener et al., 2016; Zeilig et al., 2012). As a consequence, SCI can result in evoked and spontaneous neuropathic pain that may last for the lifespan of patients.

Gamma-aminobutyric acid (GABA) plays an important role in modulation of sensory and nociceptive synaptic transmission. GABA, a neurotransmitter synthesized by glutamic acid decarboxylase (GAD), produces inhibitory synaptic outputs via binding to mainly $GABA_A$ and $GABA_B$ receptors (Barber et al., 1982; Bowery et al., 1984; Schlichter et al., 1984; Yates and Taberner, 1975). Dysfunctional GABAergic output contributes critically to various pathophysiological disorders including, but not limited to, pain, anxiety/depression, stress, and epilepsy (Elekes et al., 1986; Francois et al., 2017; Lau and Vaughan, 2014). Research findings from rodent SCI models over the last few decades support a key role of decreased spinal GABAergic inhibitory output in the

development of hyperexcitability of spinal dorsal horn neurons and chronic neuropathic pain states (Drew et al., 2004; Gwak et al., 2008; Gwak et al., 2006). This plasticity can be the summation of spatial and temporal modulations of the GABAergic system in response to SCI. In this chapter, we summarize the current understanding of spinal GABAergic mechanisms on SCI-induced neuropathic pain based on findings from rodent SCI model studies.

2. GABA Synthesis and Reuptake

In the spinal dorsal horn, GABA acts as a "counterbalance" transmitter against nociceptive transmission. There are two rate-limiting GABA-synthesis enzymes, glutamic acid decarboxylases (GADs). GAD65 (65 kDa) is mainly membrane-associated, and preferably regulates vesicular GABA release by exocytosis in the nerve terminal; whereas GAD67 (67 kDa) is mainly cytosolic and preferably regulates cytosolic GABA release (Erlander et al., 1991; Feldblum et al., 1995). Due to their differential localizations, GAD65 plays a major role in regulating rapid and focal synaptic inhibition on postsynaptic neurons whereas GAD67 mainly contributes to tonic control of neuronal activity (Feldblum et al., 1993).

Under normal conditions, GABA released from the terminals of GABAergic neurons mainly binds to $GABA_A$ and $GABA_B$ receptors to produce an inhibitory tone in synaptic transmission. However, not all released GABA binds to their receptors in the synaptic and extrasynaptic clefts. The concentration of extracellular GABA and actions of GABAergic transmission in the synaptic cleft are controlled by GABA re-uptake through high affinity GABA transporters (see detail in 6.4 GABA Transporters)

expressed in GABAergic neurons and surrounding glial cell, especially astrocytes (Chatton et al., 2003; Schousboe et al., 2004). Following GABA uptake into astrocytes, GABA-glutamate-glutamine conversions are controlled via glutamate transaminase and glutamine synthetase, respectively. After SCI, in addition to GABA uptake, glial glutamate transporters EAAT1 (GLAST) and EAAT2 (GLT1) are increased in activated astrocytes, which can facilitate glutamate uptake and lead to an increased conversion to glutamine via glutamine synthetase in the spinal dorsal horn (Broer et al., 2004; Vera-Portocarrero et al., 2002). In addition, spinal contusion and transection injuries can increase glutamine synthetase expression and activity in injured spinal dorsal horn (Benton et al., 2000; Liu et al., 2013). As a result, the final product glutamine can easily pass to neighboring GABAergic neurons and serve as a substrate for re-production of GABA depending on relative activities of phosphate-activated glutaminase (PAG) and glutamic acid decarboxylase (GAD). Inhibition of glial glutamate transporters (GLT-1) or glial glutamine synthetase causes a reduction in GABAergic synaptic transmission shown as decreased inhibitory post-synaptic currents (IPSCs) in the spinal dorsal horn (Jiang et al., 2012). This feedback mechanism is an important control of GABA homeostasis and is called "GABA-glutamate-glutamine cycle" (Schousboe et al., 1993; Struzynska and Sulkowski, 2004). Thus, expression of GABA transporters and activation of glutamine synthetase in astrocytes are important factors for GABA reuptake and resynthesis.

3. GABA Receptors

In the nervous system, three subtypes of GABA receptors have been identified to play a major role in mediating inhibitory synaptic transmission, mainly via two subtypes

of ionotropic ligand-gated $GABA_A$ and $GABA_C$ receptors and one subtype of G-protein coupled metabotropic $GABA_B$ receptors. In the spinal dorsal horn, $GABA_B$ receptors outnumber $GABA_A$ receptors (Bowery et al., 1987). We have reported previously that $GABA_A$ and $GABA_B$ receptor activation shows no significant difference on the inhibition of dorsal horn neuronal hyperexcitability and mechanical allodynia in spinal hemisection models (Gwak et al., 2006), but the relative inhibitory effects of these GABA receptors on SCI-induced neuropathic pain are not thoroughly investigated. Presynaptic activation of ionotropic ligand-gated $GABA_C$ receptors (Dong et al., 1994; Qian and Dowling, 1993) results in anti-thermal hyperalgesia to noxious heat stimulation, suggesting an inhibitory role of $GABA_C$ receptors on acute pain signal transduction under physiological conditions (Tadavarty et al., 2015). In addition, inhibition of $GABA_C$ receptors by microinjection of antagonist results in enhanced mechanical allodynia in the spared nerve injury model (Chu et al., 2020). However, the role of $GABA_C$ receptors on neuropathic pain development/maintenance has not been studied yet in SCI animals.

3.1 $GABA_A$ Receptor

$GABA_A$ receptors trigger influx of Cl^- ions and cause hyperpolarized membrane potentials, resulting in decreased neuronal firing activities in postsynaptic neurons (Strata, 1986). In electrophysiological studies, $GABA_A$ receptors are involved in two different synaptic inhibition mechanisms depending on the composition of subunits (Khan et al., 1996; Pencheva et al., 1991). $GABA_A$ receptors consisting of delta (δ) subunits and associated α and β subunits are predominantly localized extrasynaptically

and mediate persistent inhibition (tonic inhibition) (Brickley et al., 1996; Saxena and Macdonald, 1994). $GABA_A$ receptors consisting of gamma (γ) subunits and associated α and β subunits are predominantly localized at postsynaptic and extrasynaptic membranes, and regulate quantal release-mediated rapid and intense inhibition (phasic inhibition) (Nusser and Mody, 2002). In whole cell current studies, αβδ complexes display a higher affinity (20% higher) for GABA compared to δ alone, and αβγ complexes display higher (36%) responses to GABA compared to αβδ complexes. In GABA-evoked current studies, αβγ complexes display desensitization and rapid recovery to repeat GABA application whereas αβδ complexes show little desensitization and slow recovery (Saxena and Macdonald, 1994). In uninjured or non-painful conditions, blockade of $GABA_A$ receptors by $GABA_A$ receptor antagonist bicuculline results in increases of dorsal horn neuronal activity and pain behaviors in animal models (Gwak et al., 2006; Sorkin et al., 1998). These results suggest that spinal $GABA_A$ receptors play a critical role in tonic inhibition of synaptic transmission under non-painful conditions. Following spinal transection injuries, GABAergic interneurons in lamina I of spinal dorsal horn show increased tonic firing patterns including burst, frequency, and amplitudes with more depolarized membrane potentials. In addition, persistent inward currents (PICs) are observed in cells from spinal transects (Dougherty and Hochman, 2008). However, the increase of tonic inhibition can lead to the decrease of phasic inhibition in order to maintain homeostatic balance of $GABA_A$-mediated inhibition (Wu et al., 2013). Therefore, further studies are needed to clarify the differences in contributory roles of tonic and phasic inhibitions on neuropathic pain development/maintenance after SCI.

3.2 $GABA_B$ Receptor

$GABA_B$ receptors are composed of $GABA_{B1}$ and $GABA_{B2}$ subtypes, and the latter predominantly activates G-protein coupled pathways (Pin et al., 2004). Activation of $GABA_B$ receptors triggers efflux of potassium ions and prevents neuronal firing activity via inwardly-rectifying K^+ channels (GIRK) (Brewer and Baccei, 2018). Activation of $GABA_B$ receptors also prevents voltage-gated Ca^{2+} channel-mediated glutamate release at the primary afferent terminals (Strata, 1986). In this regard, high voltage-gated calcium channels are more sensitive than low voltage-gated calcium channels in modulation by inhibitory G-protein pathways (Huang et al., 2015). Intracellularly, activation of $GABA_B$ receptors inhibits cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) and cAMP-response element-binding protein (CREB) signaling pathways. Inhibition of these intracellular downstream pathways inhibits phosphorylation-dependent pathways in nociceptive signaling (Zhou et al., 2017). In addition, activation of $GABA_B$ receptors inhibits production of nitric oxide (NO), an important substrate for spinal nociceptive signaling, which attenuates neuropathic pain states after spinal transection injury in rats (Kisucka et al., 2015).

4. GABAergic Synapses

In the spinal dorsal horn, GABAergic output is mediated by GABAergic descending pathways originated from supraspinal levels and spinal GABAergic interneurons. The GABAergic terminals from supraspinal GABAergic projection

neurons, such as that in rostral ventral medulla (RVM)/periaqueductal gray (PAG), and spinal GABAergic interneurons simultaneously or individually form synapses with postsynaptic projection neurons, such as spinothalamic tract (STTs) neurons and major nociceptive-mediating neurons (Cho and Basbaum, 1991; Todd et al., 1992). GABAergic interneurons, mainly located in the superficial laminae I/II of spinal dorsal horn, also project to near and remote laminae regions via their terminals and extended dendrites that can contribute to local inhibitory circuits in sensory signal transmission (Spike and Todd, 1992). This is support by findings from numerous studies, including that from a recent investigation showing that peripheral Aβ fiber stimulation causes glutamate-mediated monosynaptic excitatory postsynaptic currents in spinal dorsal horn laminae III-IV that can be reversed by $GABA_B$ receptor agonist baclofen (Salio et al., 2017). Therefore, activation of presynaptic $GABA_B$ receptors can prevent glutamate release and neuronal activity at differential spinal laminae in the dorsal horn. Together, these results support that GABAergic inhibition in synaptic transmission is mediated by presynaptic as well as postsynaptic mechanisms.

GABAergic axons generally form synapses with dendrites or soma of postsynaptic neurons in the spinal dorsal horn, which are classified as axodendritic or axosomatic synapses. This synaptic coupling results in inhibition of firing activity in spinothalamic track (STTs) neurons followed by a decrease of nociceptive transmission (Carlton et al., 1992; Ribak and Roberts, 1990). GABAergic axons also form synapses with the terminals of GABAergic projections (axo-axonic synapses) that can inhibit their firing activity and decrease inhibitory outputs. Consequently, this inhibitory outcome can

lead to facilitation of firing activity of spinal dorsal horn neurons and enhancement of nociceptive transmission, which is called GABAergic disinhibition (Lu et al., 2008; Sivilotti and Woolf, 1994; Viguier et al., 2012). Data from neuroanatomical studies, however, show that GABAergic axons mainly form synapses with dendrites/soma of postsynaptic neurons (axosomatic synapse) and primary afferent terminals originated from DRG sensory neurons (axoaxonic synapse) in rat spinal laminae I to III (Magoul et al., 1987). Thus, the synaptic coupling between presynaptic releases of GABA and its binding at dendritic/somatic terminals of postsynaptic neurons is mainly responsible for the inhibitory tone of synaptic transmission in spinal dorsal horn. Disruption of this inhibitory pathway under pathological conditions can facilitate the development of nociception (Sawynok, 1987). Taken together, these results support that synaptic specificity of GABAergic terminals on ascending projection neurons or GABAergic interneurons is important in determining the local inhibitory or excitatory tone in nociceptive transmission.

5. GABA and Neuropathic Pain after SCI

The predominant event for neuropathic pain development after SCI is neuronal hyperexcitability in the spinal dorsal horn. Although the somatotopic structure of spinal dorsal horn is well organized to handle the complexity of sensory signal transduction with diverging but integrated components varying in spinal laminae, soma size, neurochemical classifications, encoding abilities (tactile vs. nociceptive) and projecting locations, the majority of dorsal horn neurons become hyperexcitable after SCI (Gwak et al., 2013; Hains et al., 2002). Neuronal hyperexcitability, characterized by enhanced and

long-lasting neuronal firing frequency and amplitude with decreased firing thresholds, is a critical factor contributing to the sensitization of spinal dorsal horn neurons in nociceptive transmission and neuropathic pain development/maintenance after SCI. Although SCI causes a transient increase of extracellular GABA concentration via decreased GABA reuptake and increased GAD activity as a counter-balance to SCI-induced high glutamate concentration, the duration of GABA increase is shorter than the periods of glutamate increase (Diaz-Ruiz et al., 2016; Mills et al., 2001). However, data from electrophysiological studies have shown that spinal or systemic enhancement of GABAergic outputs attenuates spinal dorsal horn neuronal firing and neuropathic pain states after SCI in animal models (Gwak et al., 2006; Hama and Sagen, 2012; Hao et al., 1991), supporting that SCI causes the loss of spinal GABAergic inhibitory outputs (Table 1).

6. Hypofunctional GABAergic Outputs on SCI Pain

Data from several transplantation studies are reported to elucidate spinal GABAergic mechanisms on SCI-induced neuropathic pain. First, subcutaneous inoculation of GAD67-expressing, replication-incompetent herpes simplex virus (HSV) vectors or replication-defective human foamy virus (HFV) vectors (rdvGAD67), at the hindpaw plantar surface attenuated spinal hemisection-induced neuropathic pain states via increased GABA release and inhibition of calcitonin gene-related peptide (CGRP) expression in the spinal cord and DRG neurons (Liu et al., 2004; Liu et al., 2008). Second, transplantation of the human neuronal NT2 cell line (hNT2.17 cells), which releasees GABA, into subarachnoid space reversed neuropathic pain states in the

quisqualic acid (QUIS, the AMPA-metabotropic receptor agonist)-induced excitotoxic SCI rats (Eaton et al., 2007). Third, transplantation of mouse embryonic stem cell (ESC)-derived GABAergic neurons into subarachnoid significantly reduced neuropathic pain states and hyperexcitability of spinal wide dynamic range (WDR) neurons (a subclass of spinal dorsal horn neurons capable of increasing response intensity and receptive field to a large range of stimuli) without affecting serotonergic function (another major spinal inhibitory neurotransmitters) in a spinal hemisection injury model (Kim et al., 2010). Intraspinal transplantation of embryonic GABAergic neural precursor cells (NPCs) into the injured spinal dorsal horn also reduced excitotoxic-induced neuropathic pain states with increased NeuN-positive GABA-immunoreactivity (Lee et al., 2012). In addition, intrathecal treatment with mouse embryonic stem cell derived neural precursor cells (mESC-NPCs) increased spinal GABAergic neurons and behavioral thresholds (is this what you mean?) for mechanical stimulation at the hindpaw and body trunk in contusion SCI rats (Hwang et al., 2016). Findings from these behavioral, immunohistochemical, and electrophysiological studies strongly support that the loss of spinal GABAergic inhibitory tone contributes critically to the development/maintenance of SCI-induced neuronal hyperexcitability and neuropathic pain states.

Many factors may lead to hypofunctional GABAergic inhibitory output in the spinal cord, which is an important contributor to the underlying mechanisms of SCI-induced neuropathic pain states as addressed later.

6.1 Loss of GABAergic Neurons

Loss of GABAergic neurons in the spinal dorsal horn has been well demonstrated in various types of SCI animal models. The moderate T11 contusion injury results in waves of cytotoxic events followed by a loss of GABAergic interneurons in the lumbar superficial dorsal horn, which are mainly innervated by primary C and Aδ nociceptive fibers (Meisner et al., 2010). In the excitotoxic injury model, QUIS injection at T12 to L1 causes a decrease of GABAergic neurons in the superficial dorsal horn, and intraspinal transplantation of embryonic NPCs restores NeuN-positive GABAergic neurons and process densities to normal levels (Dugan et al., 2020). Spinal cord ischemia-reperfusion injury (SCII) also results in a decrease of GAD65 in the axon terminals with a loss of Nissl bodies in the spinal cord (Yu et al., 2014). These findings support that SCI causes the loss of GABAergic distribution in the spinal dorsal horn.

One of the mechanistic events for the loss of GABAergic neurons in the spinal dorsal horn after SCI is the excitotoxicity-mediated inflammation and apoptosis. SCI increases excitotoxic level of glutamate, proinflammatory cytokines, and reactive oxygen species (ROS) (Gwak et al., 2013; Hassler et al., 2014; Sabirzhanov et al., 2019) that leads to secondary pathophysiological processes such as central cavitation, apoptosis and vascular/membrane derangements (Oyinbo, 2011). In excitotoxic events, SCI can cause opposite two-way effects that may contribute to the neuronal loss. For example, SCI can increase the level of apoptotic gene activators, NK-kB p65/p50, and decrease the level of antiapoptotic gene activator, c-Rel, in neurons (Rafati et al., 2008). NK-kB p65/p50 promotes the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) that contribute to neuronal cytotoxicity via oxidative stress, protein S-

thiolation, lipid peroxidation, nucleic acid degradation as well as inflammation. Thus, SCI-induced apoptotic pathway activation and subsequent increases of ROS and inflammation can induce GABAergic neuronal death in lamina I-III (Im et al., 2012; Sah et al., 2002; Sharma and Sjoquist, 2002). Proinflammatory cytokines and ROS can also activate tumor necrosis factor receptor 1 (TNFR1) and initiate activation of the cysteine proteinase family, such as caspases 3 and 8, that lead to apoptosis of GABAergic interneurons in the spinal dorsal horn (Gavilan et al., 2007; Santo-Domingo et al., 2015). Activation of rho-associated protein kinase 2 (ROCK2) after SCI increases the activity of caspase-1 and tumor necross factor-α (TNF-α), known mediators of inflammation and cell death, which may also contribute to a loss of GABAergic interneurons (Nimgampalle et al., 2019). In addition, inoculation of gad1 gene (typically expresses GAD67) through replication-defective herpes simplex virus (HSV) vectors inhibits the signaling pathway of ROS-mediated intracellular transcriptional phosphorylated cAMP response element-binding protein (pCREB) in mitochondria and increases GABAergic immunoreactivity in the spinal dorsal horn (Kanao et al., 2015). SCI also causes caspase-3 activation in the spinal superficial dorsal horn that results in a selective loss of GABAergic neurons as well as decreased expression of GAD65 and GAD67 in a SCI-induced neuropathic pain model (Meisner et al., 2010). Taken together, these data support that SCI induces apoptotic processes via inflammation and oxidative stress that lead to the loss of spinal GABAergic neurons in spinal dorsal horn.

6.2 Decrease of GABA Synthesis

Even though GABAergic neuronal death is a contributing factor to the decrease of GAD expression, GAD downregulation may also occur without the loss of GABAergic neurons. After SCI, activated primary afferent fibers may activate GABAergic interneurons and subsequently initiate the release of GABA into the extracellular space to counterbalance glutamate-mediated excitotoxicity (Diaz-Ruiz et al., 2016; Liu and McAdoo, 1993). This can trigger GABA reuptake from the extracellular space into activated astrocytes through GABA transporters, thus resulting in the conversion to glutamate and glutamine intracellularly (Leke and Schousboe, 2016; Qureshi et al., 2020). This leads to increased astrocytic glutamine efflux via amino acid transport systems and its uptake into neighboring GABA neurons by system A transports (Chaudhry et al., 2002) where it serves as a substrate for GABA synthesis again. These findings support that astrocytic glutamine is a major source of GABA re-synthesis (recycling) in GABAergic neurons (Patel et al., 2001). As described earlier, GABA reuptake pathways can activate GAD, but high concentration of intracellular GABA can also inhibit the activity of GAD. It has been shown that contusion or chemical-induced SCI leads to downregulations of the GABA synthesizing enzymes (GAD65/67), which results in spinal disinhibition, persistent central sensitization and neuropathic pain states (Berrocal et al., 2014; Deumens et al., 2013). In addition, glutamate and other nociception-stimulating agents, such as proinflammatory cytokines, ROS and ATP, can activate intracellular downstream events and modulate specific gene expression that result in downregulation of GAD expression (Gwak and Hulsebosch, 2011).

6.3 Decrease of Primary Afferent Innervation

SCI may lead to damages of primary afferent fiber at, near the injury side and in the remote regions of spinal cord, which is followed by the loss or degeneration of primary afferent fibers (so called dying back phenomenon). Subsequent disconnections of synaptic coupling between primary afferent fibers and GABAergic interneurons can lead to a decrease of GABAergic output in spinal inhibitory circuits. The loss of GABAergic inhibitory output often causes facilitation of subthreshold primary afferent Aδ and unmyelinated C fiber inputs during nociceptive transmission (Lu et al., 2008). Normally, subthreshold primary afferent input gradually decays due to weak input activity and the lack of conductance that fail to trigger action potential propagation or activation of postsynaptic neurons in the spinal dorsal horn (Takazawa and MacDermott, 2010). However, the loss of GABAergic inhibitory outputs after SCI can facilitate a temporal and spatial summation of subthreshold primary afferent activation, which can lead to action potential generation and propagation as well as enhanced nociceptive transmission in the spinal dorsal horn.

In a recent study, Mazzone and Nistri have reported that extrasynaptic $GABA_A$ receptors play a critical role in neuroprotection by counteracting glutamate-mediated excitotoxicity (Mazzone and Nistri, 2019). In addition, an enhancement of $GABA_B$ receptor activity after SCI inhibits axonal degeneration via inhibition of glutamate-mediated excitotoxicity and caspase activity (Romaus-Sanjurjo et al., 2018). Others have also reported that GABA plays a role as an endogenous regenerative factor in the CNS.

For examples, treatments with $GABA_A$ or $GABA_B$ receptor agonist Propofol or Baclofen inhibit glutamate-mediated excitotoxicity and caspase activation after CNS injury in rats (Han et al., 2008; Kaur et al., 2016; Lopez-Bendito et al., 2003). These results suggest that enhancement of GABAergic inhibitory outputs maybe an important factor in restoration of primary afferent fiber innervation in the spinal dorsal horn.

Activation of calcium-dependent phospholipase 2 (cPLA2) is critically involved in axonal demyelination following SCI-induced axonal damages (Khan et al., 2015). It has been reported that pharmacological blockage or genetic deletion of cPLA2 reverse neuronal and axonal damages after SCI via inhibition of pERK activity and lysosomal damages, suggesting that SCI-induced activation of cPLA2 contributes to axonal damages through a pERK-dependent pathway that may lead to subsequent formation of maladaptive synapse circuits in the spinal dorsal horn (Li et al., 2019; Liu et al., 2014). In addition, activation of calcium-independent phospholipase 2 (iPLA2) also contributes to axon terminal degeneration via damages of mitochondria in an axonal degeneration mouse model that results in the loss of presynaptic innervation (Sumi-Akamaru et al., 2015). Taken together, these findings support that activation of PLA2 and oxidative damages contribute to axonal damages and subsequent loss of afferent fiber innervation on GABAergic neurons after SCI that may lead to subsequent malformation of synaptic circuits in the spinal dorsal horn and development of neuropathic pain states.

6.4 GABA Transporters

One pathway for clearance of extracellular GABA and termination of GABA-mediated synaptic transmission is the removal of extracellular GABA by GABA

transporters (GATs). In general, neuronal $GABA_A$ and glial $GABA_B$ GATs act as a key player to maintain the appropriate extracellular level of GABA and to terminate GABAergic synaptic transmission (Jones and Neal, 1976; Schon and Kelly, 1975). GATs are divided into five different transporter categories: Vesicular GABA transporters (VGAT) are mainly expressed at the nerve ending (Chaudhry et al., 1998) and four subtypes of Na^+-dependent transporters are expressed in human and rat; GAT1 (Guastella et al., 1990), GAT2/GAT3 (Borden et al., 1992) and a low affinity GABA transporter BGT1 (Yamauchi et al., 1992). In mice, GAT1, BGT1, GAT2, and GAT4 correspond to GAT1, GAT2, GAT3, and BGT1 in human and rat, respectively (Zhou and Danbolt, 2013). In the spinal cord, GAT1 is highly colocalized with GABA or GAD, suggesting that GAT1 mainly contributes to presynaptic inhibition. GAT3 is highly expressed in astrocytes. Both GAT1 and GAT3 transporters are localized near synaptic clefts and play an important role in regulating extracellular GABA concentrations and terminating GABAergic synaptic actions (Borden, 1996; Scimemi, 2014). BGT1 is highly expressed extrasynaptically on both neurons and glial cells. These distribution patterns suggest that the heterogeneity of GATs is critical in mediating site-specific GABA binding and termination of GABA's actions.

Overproduction of GATs in neurons or astrocytes accelerates GABA uptake that may result in a loss of extracellular GABA in spinal synaptic circuits and lead to hyperexcitability of neurons. For example, genetically enhanced or decreased GAT1 expression results in hyperalgesic or hypoalgesic conditions, respectively (Ref?). In addition, GAT1 inhibitors (NO-711 and tiagabine) can attenuate spontaneous and evoked pain behaviors in acute inflammatory pain models (Hu et al., 2003). However, Meisner et

al. have reported that SCI-induced decrease of GAT-1 expression, probably due to a loss of GABAergic neurons, in spinal dorsal horn results in decreased GAD activity and GABAergic inhibitory outputs that correlate well with neuropathic pain states in the chronic phase (over a month after SCI) (Meisner et al., 2010). Interestingly, the expression level of GAT3 in the lumbar segment is not affected by mid thoracic SCI, suggesting that SCI-induced changes of GAT expression is injury region specific (Pallottie et al., 2018). These findings suggest that SCI-induced dysregulation of GATs can be regional-specific, and affect the availability of GABA, which in turn can disrupt normal GABAergic outputs in the spinal dorsal horn and contribute to neuropathic pain states. The roles and regulations of GAT2 and BGT1 are not sufficiently studied under SCI-induced neuropathic pain conditions.

6.5 Cation-Chloride Cotransporters

It is well known that excitatory or inhibitory synaptic transmission are mainly mediated by neurotransmitters/neuropeptides and expression/activity levels of their receptors on the cell membrane. However, chloride ions can cause membrane potential changes via a chloride-gradient crossing the cell membrane that is also important in regulating $GABA_A$ receptor-mediated functions (Kaila, 1994). Two chloride transporters have been discovered so far to control the homeostasis of Cl^- concentration in the spinal cord: Na^+-K^+-Cl^- cotransporter 1 (NKCC1) transports Cl^- into the cell; whereas K^+-Cl^- cotransporter 2 (KCC2) transports Cl^- from the cytosol to extracellular space (Misgeld et al., 1986; Payne et al., 2003). Therefore, NKCC1 increases intracellular Cl^- concentration whereas KCC2 decreases intracellular Cl^- concentration (Takazawa and MacDermott,

2010) that regulates transmembrane Cl^- gradients.

Notably, it has been reported that SCI causes upregulation of NKCC1 and downregulation of KCC2 in the spinal cord that correlate with neuropathic pain states (Hasbargen et al., 2010). These bi-directional changes of chloride transporters can result in a high concentration of intracellular Cl^- that often leads to changes in the reversal potential of GABA responses (E_{GABA}), resulting in $GABA_A$ receptor-mediated efflux (excitatory), instead of inflex (inhibitory), of Cl^- across the membrane and causes membrane depolarization (van den Pol et al., 1996). This change from inhibitory to excitatory transmembrane Cl^- potentials triggers a switch of GABA function (ionic plasticity) from neuronal inhibition to excitation, which is believed to play a critical role in mediating enhanced nociceptive transmission and chronic neuropathic pain development following SCI (Grau and Huang, 2018; Lu et al., 2008)

6.5.1 NKCC1

Findings from functional studies with NKCC1 knockout mice support that NKCC1 appears to be the primary transporter playing a dominate role in inward Cl^- transport and responsible for the active accumulation of intracellular Cl^- above the electrochemical potential equilibrium in DRG neurons. As a result, GABA (through $GABA_A$ receptors) can trigger a depolarizing Cl^- efflux (mainly excitatory) in sensory neurons, in contrast to triggering a hyperpolarizing Cl^- influx (inhibitory) in other mature neurons with a low intracellular Cl^- concentration (Sung et al., 2000, JNS).

Presynaptically, the high concentration of intracellular Cl^- in the terminals of nociceptors such as Adelta and C-fibers often causes Cl^- efflux-mediated small primary afferent depolarization (PADs) upon $GABA_A$ receptor activation by GABA from GABAergic interneurons (Lin et al., 2000; Payne et al., 2003; Sung et al., 2000), which may receive synaptic input from low-threshold mechanoreceptive fibers (Aβ). Under normal (or physiological acute pain) conditions, activation of GABAergic interneurons, for example, by Aβ stimulation can lead to these sub-threshold PADs that can cause inactivation of Na^+ channel activities, interruption of orthodromic propagation of action potentials and result in decreased excitatory neurotransmitter release from nociceptive afferent terminals, a phenomenon called presynaptic inhibition (Brumback and Staley, 2008) (Pitcher and Cervero, 2010; Willis, 2006).

However, under pathological conditions, such as SCI, increased activity and expression of NKCC1 (Cramer et al., 2008) (Hasbargen et al., 2010) (Ahmed et al., 2014; Lee et al., 2014) can enhance accumulation of PADs that may lead to a larger depolarization above the threshold for action potential generation. This in turn, can cause maladaptive propagation of action potentials and exaggerated excitatory neurotransmitter release, which can activate postsynaptic dorsal horn neurons such as excitatory projection neurons and WDR neurons (Aptel et al., 2007; Pitcher and Cervero, 2010; van den Pol et al., 1996) and result in nociceptive transmission (Keller et al., 2007) (Pitcher et al., 2010) (Bardoni et al., 2013; Willis, 2006). This NKCC1-mediated loss of GABAergic presynaptic inhibition, or called disinhibition, is believed to contribute to dorsal horn neuron sensitization and development of neuropathic pain states after SCI as

demonstrated in multiple SCI models. For example, contusion SCI causes increased NKCC1 expression in both early and late phases that correlates with the development of thermal hyperalgesia, and treatments with NKCC1 inhibitor bumetanide attenuate thermal hyperalgesia (Cramer et al., 2008) (Hasbargen et al., 2010). In addition, contusion SCI leads to increased activity of NKCC1 due to increased NKCC1 phosphorylation in injured spinal cord neurons, which is likely regulated by injury-induced activation (phosphorylation) of a neuronal isoform (250 kDa) of with-no-lysine kinase 1 (WNK1), a known upstream regulator of NKCC1 (Ahmed et al., 2014; Lee et al., 2014). Together, these results support that the NKCC1-WNK1 signaling pathway contributes to the development and/or maintenance of neuropathic pain states following SCI.

6.5.2 KCC2

The downregulation of KCC2 (Boulengues et al., 2010) (Huang et al., 2016) followed by decreased GABAergic inhibitory output contributes to central sensitization and neuropathic pain states after SCI (Lu et al., 2008). Downregulation of KCC2 leads to increased intracellular Cl^- concentrations and development of E_{GABA} that can generate Cl^- efflux, instead of influx, upon membrane depolarization due to $GABA_A$ receptor activation, and causes neuronal excitation. Thus, E_{GABA} prevents the inhibitory synaptic transmission and promotes the generation and propagation of primary A and C fiber-mediated action potentials in the spinal dorsal horn, leading to neuropathic pain development post SCI (Grau and Huang, 2018; Lu et al., 2008). This is supported by

findings that drug treatments (with bumetanide, for example) that can restore low intracellular Cl^- concentrations and GABAergic inhibition can abolish SCI-induced neuropathic pain states (Cramer et al., 2008; Hasbargen et al., 2010). Together, these findings support that decreased expression of KCC2 contributes to diminished GABAergic inhibitory outputs and triggers the switch of GABAergic inhibition to excitation, leading to spinal sensitization and neuropathic pain development after SCI (Huang et al., 2016).

Mechanistically, SCI-induced downregulation of brain-derived neurotrophic factor (BDNF) may be responsible for the decreased KCC2 expression and subsequent disinhibition (Beverungen et al., 2020; Grau et al., 2014; Huang et al., 2017) since BDNF treatment increases the expression of KCC2 and restores GABAergic inhibitory outputs (Hains et al., 2002). Furthermore, normal KCC2 activity is also critical in maintaining GABAergic inhibition, which is supported by findings from a recent study that treatment with adeno-associated virus vectors encoding neurotrophic factor (NT)-3 (AAV-NT3) restores KCC2 activity and render neuroprotection of GABAergic interneuron in the spinal cord after contusive SCI (Chang et al., 2019).

Taken together, a large body of existing evidence supports that shift of Cl^- gradient in GABAergic neurons can play a key role in regulating $GABA_A$ receptor-mediated inhibitory or excitatory synaptic sensory transmission under pathophysiological conditions, including SCI.

Summary

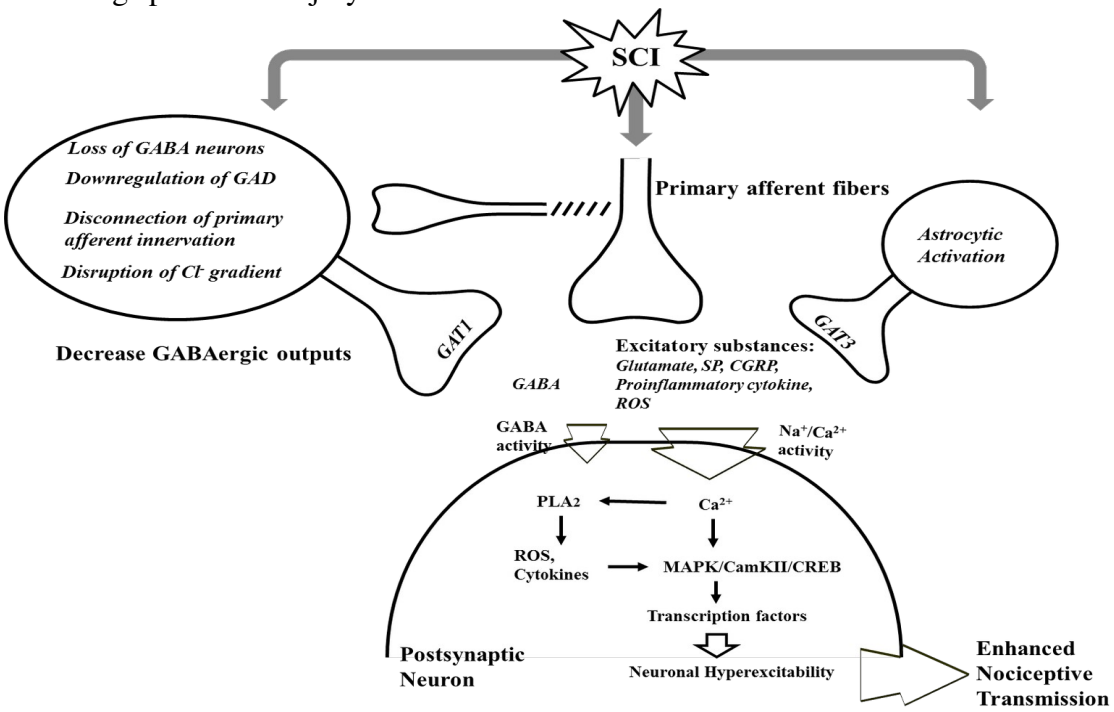
In this book chapter, we have focused on the role of spinal GABAergic mechanism on chronic neuropathic pain development following SCI. SCI often causes synaptic reorganization and disruption of spinal inhibitory tones. Consequently, an imbalance between excitatory and inhibitory output in synaptic transmission results in enhanced nociceptive transmission. While neurons and glial cells synergistically modulate GABAergic homeostasis in synaptic transmission via regulation of transporter expressions and GAD activities, SCI can cause the loss of GABAergic neurons, downregulation of enzymes critical for GABA synthesis, disconnection of primary afferent innervation onto GABAergic interneurons, and shift of Cl^- homeostasis. All these changes can alter GABA production, release, distribution, and recycle that eventually can contribute to neuronal hyperexcitability and chronic neuropathic pain states (Figure 1). Therefore, enhancing or restoring spinal GABAergic inhibitory outputs by selective pharmacological or molecular approaches can be a valuable therapeutic intervention strategy to alleviate persistent central neuropathic pain following SCI.

Table 1. The changes of spinal dorsal horn neuronal firing activity following GABA receptor activation in SCI-induced neuropathic pain conditions.

	Topical application of muscimol (1 μg), number of impulses/sec						
		Vehicle (n = 5)		*Ipsilateral* (n = 6)		*Contralateral* (n = 8)	
	Sham	*Before*	*After*	*Before*	*After*	*Before*	*After*
Brush	11.4 ± 1.5	38.1 ± 5.3	40.4 ± 4.3	34.2 ± 8.6	14.2 ± 8.6*	30.9 ± 4.9	19.7 ± 6.0
Pressure	14.9 ± 1.8	30.3 ± 4.8	31.3 ± 5.5	43.5 ± 12.6	8.1 ± 3.3*	35.9 ± 3.9	16.9 ± 5.5*
Pinch	20.3 ± 1.6	47.3 ± 6.3	44.7 ± 5.1	60.3 ± 14.3	18.3 ± 8.1*	50.3 ± 6.6	21.1 ± 6.1*
	Topical application of baclofen (0.1 μg), number of impulses/sec						
		Vehicle (n = 5)		*Ipsilateral* (n = 6)		*Contralateral* (n = 8)	
	Sham	*Before*	*After*	*Before*	*After*	*Before*	*After*
Brush	11.9 ± 1.7	31.6 ± 6.1	29.9 ± 5.3	27.8 ± 4.1	15.7 ± 3.8*	34.1 ± 2.8	18.9 ± 4.6*
Pressure	15.8 ± 1.6	28.5 ± 4.7	25.6 ± 6.5	38.8 ± 5.7	12.5 ± 5.5*	26.4 ± 2.3	16.2 ± 3.2*
Pinch	20.9 ± 1.4	42.5 ± 5.3	39.7 ± 7.1	51.0 ± 6.3	17.8 ± 7.7*	54.6 ± 5.1	20.8 ± 5.4*

The wide dynamic range (WDR) neuronal firing rates (impulse/sec) showed significant increases at both ipsilateral (injured side) and contralateral (uninjured side) spinal dorsal horn after unilateral spinal cord injury. However, the activation of spinal GABA receptors via topical application (injected onto spinal surface directly) of muscimol (GABAA receptor agonist) and baclofen (GABAB receptor agonist) significantly attenuated the firing activity. Brush: smoothly brushing the receptive field skin with soft brush, Pressure: firm pressure, Pinch: painful pressure. Neuronal firing rates were recorded 10 min after topical application using *in vivo* extracellular recording. *$p<0.05$ compared to before. Impulses activity was represented by spikes/second. Modified from Gwak et al., 2006.

Figure 1. Schematic diagram for the spinal dorsal horn neuronal hyperexcitability following spinal cord injury



Following SCI, the activated primary afferent fibers and astrocytes simultaneously released excitatory substances that trigger activation of postsynaptic neurons via sodium and calcium-mediated processes. However, SCI also reduced GABAergic outputs via loss of GABA neurons, downregulation of GAD, disconnection from primary afferent fiber innervations, and altered chloride gradients. In addition, neuronal (GAT1) and glial (GAT3) GABA transporters facilitate the GABA reuptake that results in the decrease of extracellular GABA concentration. The smaller (*thin arrow*) GABAergic inhibitory activity cannot counterbalance the massive excitatory synaptic transmission (*thick arrow*) that results in neuronal hyperexcitability and enhanced nociceptive transmission. GAD: glutamic acid decarboxylase, MAPK: mitogen activated protein Kinase, CamKII: Calcium/calmodulin-dependent protein kinase II, CREB: cAMP-response element-binding protein, GAT1 and GAT3: GABA transporters 1 and 3, SP: substance P, CGRP: calcitonin gene-related peptides, ROS: reactive oxygen species, PLA2: phospholipase A2.

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