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Immunohistochemical Study of Spinal Cord Injury Induced Neuropathic Pain with GABAergic Neural Stem Cell Transplantation Treatment

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To my parents

Abbreviations

AB	Avidin biotin
ASICs	Acid-sensing ion channels
ATP	Adenosine triphosphate
BBB	Basso, Beattie and Bresnahan
BDNF	Brain-derived neurotrophic factor
CCCs	Cation-chloride cotransporters
CCI	Chronic constriction injury
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
DAB	3,3′-Diaminobenzidine
DRG	Dorsal root ganglia
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GATs	GABA transporters
GFAP	Glial Fibrillary Acidic Protein
GNSCT	GABAergic neural stem cell transplantation
HIER	Heat-Induced Epitope Retrieval
IASP	International Association for the Study of Pain
Iba-1	Ionized calcium-binding adapter molecule 1
IB4	Isolectin B4 positive
ICD	International Classification of Diseases
IL	Interleukin
KCC2	K+-Cl− cotransporter 2
MAPK	Mitogen-activated protein kinase
MSCs	Mesenchymal stem cells
NGF	Nerve growth factor
NKCC1	Na+-K+-Cl− cotransporter 1

NMDA	N-methyl-D-aspartate
NSPCs	Neural stem and progenitor cells
OECs	Olfactory ensheathing cells
OPCs	Oligodendrocyte precursor cells
PBS	Phosphate-buffered saline
PIER	Proteolytic-Induced Epitope Retrieval
RNS	Reactive nitrogen species
ROS	Reactive oxygen
SCI	Spinal cord injury
SD	Standard deviations
SNI	Spared nerve injury
SP	Substance P
TBS	Tris-buffered saline
TPBS	Tris phosphate buffered saline
TNF-α	Tumor necrosis factor alpha
TrkA	Tyrosine receptor kinase A
TRPV	Transient receptor potential cation channels
VGCCs	Voltage-gated calcium channels
VGSCs	Voltage-gated sodium channels
5-HT	Serotonin

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1. Introduction

According to the definition of the International Association for the Study of Pain (IASP), neuropathic pain is classified as a direct consequence of a lesion or disease affecting the somatosensory system, and it is generally chronic and refractory (Treede, 2010). Recent epidemiological researches have revealed that the morbidity of neuropathic pain ranged from 6.9% to 10% (van Hecke et al., 2014) and thus increased the burden of the health care system. Based on the damaged locations, the chronic neuropathic pain is classified into peripheral neuropathic pain (originating from damage to the peripheral nerve, plexus, dorsal root ganglion or root) and central neuropathic pain (originating from damage to the spinal cord, brainstem, thalamus or cortex) (Scholz et al., 2019). Actually, spinal cord injury (SCI) is one of the most important and common causes of central neuropathic pain (Scholz et al., 2019). Clinically, neuropathic pain is characterized by unpleasant symptoms such as spontaneous pain (pain without any stimulus), hyperalgesia (unusually elevated pain produced by a normal stimulus), and allodynia (pain elicited by a stimulus that usually does not provoke pain) (Jensen and Finnerup, 2014). The major clinical manifestations are shooting or burning pain, numbness, sensory changes, and very indescribable sensations (Treede, 2010).

1.1. Spinal cord injury (SCI) and neuropathic pain

The spinal cord controls the voluntary muscles of the trunk as well as upper and lower extremities, and receives sensory input from these areas of the body (Darby, 2017). The damage to the spinal cord generally results in multiple dysfunctions like neuropathic pain and impaired mobility (Cardenas and Felix, 2009, Ahuja et al., 2017). SCI accounts for about 0. 2-0. 5% of all kinds of trauma in the whole body. Epidemiologically, there are about two million newly diagnosed patients with spinal cord injury every year. Among the patients with SCI, 11% ~ 94% are identified to

have the complication of the central pain (van Hecke et al., 2014). Previously, clinical observations showed that the central pain generally occurred within 4 weeks after SCI. The central pain was usually severe and extensive and could be divided into three subtypes (the pain above /at /below the level of the injury plane). Notably, the pain below the injury plane was the most common one. The pain had various manifestations such as knife cutting pain, burning pain, stabbing pain, radiation pain, tightening pain, etc. The pain is often persistent, unbearable, extremely painful, seriously affecting the patient's daily life (Hagen and Rekand, 2015). Since the pathogenesis of post-SCI central pain still remained incompletely elucidated, so far, there is no consensus on the diagnostic and treatment standards. Currently, therapeutic approaches like physiotherapy, psychotherapy, drug therapy (non-steroidal anti-inflammatory and analgesic drugs, anti-epileptic drugs, antidepressants, opioids, etc.) and surgical interventions are utilized in the clinical practice. Nonetheless, these approaches are still limited by the low specificity, poor efficacy and the possible strong side effects of the drugs. e.g. the depression, drug addiction, or even suicide (Treede, 2010).

1.2 SCI pathophysiological processes

A basic knowledge of pathophysiological processes in the spinal cord following injury is quite essential for the comprehension and interpretation of neuropathic pain after SCI.

Spinal cord injury immediately causes hemorrhage and cell death, but the followed multiple secondary injury cascades result in delayed damage to the tissue (Witiw and Fehlings, 2015). Commonly, the spinal cord injury responses contain three basic processes, i.e. acute phase, secondary phase and chronic injury phase (Table 1).

In the acute phase (seconds to minutes after the injury), the initial mechanical and ischemic insults would lead to instantaneous destruction of neural tissue, e.g. the cell death, axons transection, and blood–spinal cord barrier disruption (Ahuja et al., 2017).

Then, those injured cells are identified to have electrolytic shifts involving the intracellular Na^+ concentrations increase, extracellular K^+ concentrations increase, and intracellular Ca^{2+} concentrations increase that contribute to a barrage of action potentials (Couillard-Despres et al., 2017). Concurrently, the accumulation of neurotransmitters is triggered by the necrosis at the injured site. Besides, the microvasculature disruption inducing the hemorrhage, thrombosis, vasospasm and edema can lead to ischemia and local hypoperfusion, and thus further aggravate the spinal cord damage (Tator and Fehlings, 1991).

The secondary phase of SCI starts within minutes and lasts for weeks, and during this process, the cell death, edema, ischemia, electrolytic shifts, and neurotransmitters accumulation continue from the acute phase. Within the first 15 minutes following trauma, glutamate, which is an excitatory neurotransmitter of the central nervous system (CNS), is massively released as a result of cell lysis (Doble, 1999). The glutamate concentration reaches the cytotoxic level and causes excitotoxic cell death. The expression of glutamate receptors is massively detected at the surface of neurons, as well as oligodendrocytes and astrocytes which make them strongly assailable to glutamate excitotoxicity (Karadottir and Attwell, 2007). Additionally, spinal cord injury and the relevant upregulated expression of glutamate could change the ionic membrane flux, resulting in a high level of intracellular Ca^{2+} and Na^+ concentrations and reducing intracellular K^+ concentrations. The elevated intracellular Ca^{2+} activates protein kinases and phospholipases, stimulates the increase of reactive oxygen (ROS) generation and leads to mitochondrial dysfunction, and ultimately mediates the cell apoptosis (Pivovarova and Andrews, 2010, Couillard-Despres et al., 2017). Furthermore, the imbalance of Na^+/K^+ level exacerbates the cell swelling and cytotoxic edema, and issues in intracellular H+ increase-induced axonal acidosis. On the one hand, free-radical production and lipid peroxidation also play key roles in the phase of secondary injury. ROS and reactive nitrogen species (RNS) formation is a sequel of glutamate excitotoxicity, Ca2+ accumulation and mitochondrial dysfunction. The formation of ROS and RNS leads to nucleus lysis, cytoplasmic organelle damage, and cell membrane disruption (Lushchak, 2014). On the other hand, the immune

system reacts to spinal cord injury instantly and lasts for several weeks to months which processes a complex impact on the outcome of trauma (Couillard-Despres et al., 2017). Multiple immune cells (e.g. neutrophils, microglia, and lymphocytes) and regulatory proteins (e.g. cytokines and the complement system compartments) participate in the inflammatory cascade (Oyinbo, 2011). Though the immune system reactions can clear cellular debris and thus play a neuroprotective role during the injury process, the overactivation of the inflammatory response is supposed to mediate cell apoptosis and aggravate tissue edema that further exacerbate the injury (Peterson and Anderson, 2014, Bastien and Lacroix, 2014). Moreover, the demyelination caused by acute oligodendrocyte loss following SCI damages the structure of the myelin layer and destroys its function. This damage will persist for months and impairs nerve signal conduction (Totoiu and Keirstead, 2005, Alizadeh et al., 2015). In addition, during the secondary phase, the astrocytes around the lesion will be activated to proliferate, which can be specifically detected by Glial Fibrillary Acidic Protein (GFAP) (Orr and Gensel, 2018). The presence of numerous astrocytes majorly composes the glial scar around the damaged region. Besides, the microglia, endothelial cells, fibroblasts, and basal membrane also take part in the scar formation (Yuan and He, 2013).

At last, in the chronic phase (months to years), apoptosis and demyelination continue from the secondary phase. The glial scar maturation and regenerative axonal sprouting are remarkable features of this phase (Hill et al., 2001). What's more, the neurocircuits and the expression of ion channels are altered, which would lead to the occurrence, development, and persistence of the chronic neuropathic pain (Christensen and Hulsebosch, 1997). The cyst formation occurs in a portion of patients with SCI, and the cyst can continue to expand over time, causing further neurological deficits against the recovery of spinal function (Krebs et al., 2016). Usually, the lesion and the associated function loss become stabilized in one or two years after injury.

Table 1. Major features of the three phases of spinal cord injury responses.

Major features of the three phases of spinal cord injury responses	
Acute Phase (Seconds to minutes after Injury)	Cell death from mechanical or ischemic insults Hemorrhage Vasospasm Thrombosis Ischemia Edema Electrolytic shifts （Na^+，K^+ and Ca^{2+}） Membrane disruption Accumulation of neurotransmitters
Secondary Phase (Minutes to Weeks)	Continued ischemic cell death Continued edema Continued ischemia Continued electrolytic shifts Continued neurotransmitters accumulation Free-radical production and lipid peroxidation Neutrophil and lymphocyte invasion and release of cytokines Microglial activation Apoptosis Demyelination Astroglial scar launch
Chronic Phase (Months to Years)	Continued apoptosis Continued demyelination Scar formation Alteration of ion channels and their receptors Formation of cyst and cavity Regenerative processes, including sprouting by neurons Alteration of neurocircuits

Note: this table is reproduced from three previous studies (Tator et al., 1995, Hulsebosch et al., 2002, Oyinbo et al., 2011).

1.3 The pathway of pain transmission

Pain transmission is a sophisticated and dynamic process that involves multiple levels of the central neural system. A variety of noxious stimuli can induce pain, such as

inflammation, compression, toxic chemicals, and thermal stimuli (Bell, 2018). The nociceptors are fiber terminals of specialized peripheral sensory neurons and the ganglia are a structure containing the cell bodies of afferent neurons. In general, the noxious stimuli are detected by nociceptors and they are transduced into electrical signals and conveyed to dorsal root ganglia (DRG) or trigeminal ganglia (Yam et al., 2018). There are three major primary afferent fibers characterized by different features (Table 2).

Table 2. Characteristics of the three major primary afferent fibers.

Fiber types	**Aβ**	**Aδ**	**C**
Diameters	6–20μm	1–5μm	0.2–0.5μm
Stimuli	Non-noxious	Non-noxious or noxious	Polymodal noxious
Myelin	Yes	Thin	No
Conduction velocity	80–120m/s	35–75m/s	0.5–2m/s
Sensory function	Touch	'Fast' pain	'Slow' pain
Dorsal Horn termination	lamina III	lamina I, IV	70% − peptidergic, TrkA +, lamina I/IIo 30% − non-peptidergic, IB4+, lamina IIi/III

Note: this table is reproduced from two previous studies (Hunt and Mantyh, 2001, Urch, 2007). Lamina IIo = outer parts of Lamina II; Lamina IIi = inner parts of Lamina II; TrkA + = tyrosine receptor kinase A positive; IB4+ = isolectin B4 positive. IB4 binding and TrkA expression can be applied to define subpopulations of C fibers.

Then, the electrical impulses travel along the primary afferents and they are propagated to the projection neurons in the dorsal horn, where the signals can be extensively modulated by intrinsic interneurons, glia, and descending pathways (Urch, 2007). Within this process, the synaptic junction transmits impulses from a

presynaptic cell to a postsynaptic neuron by conducting an action potential→ neurotransmitter→action potential signal conversion. Following this, the axons of those projection neurons form the afferent bundles that cross the midline, travel in the spinal ascending tracts, pass through the brainstem and thalamic nuclei, and reach the cerebral cortex. Afterwards, the somatosensory cortex of the brain processes the sensory information to detect the position and intensity of the noxious stimuli, and this sensation is perceived as pain (Rajneesh and Bolash, 2018).

An overview of the pain transmission route is described in Figure 1.

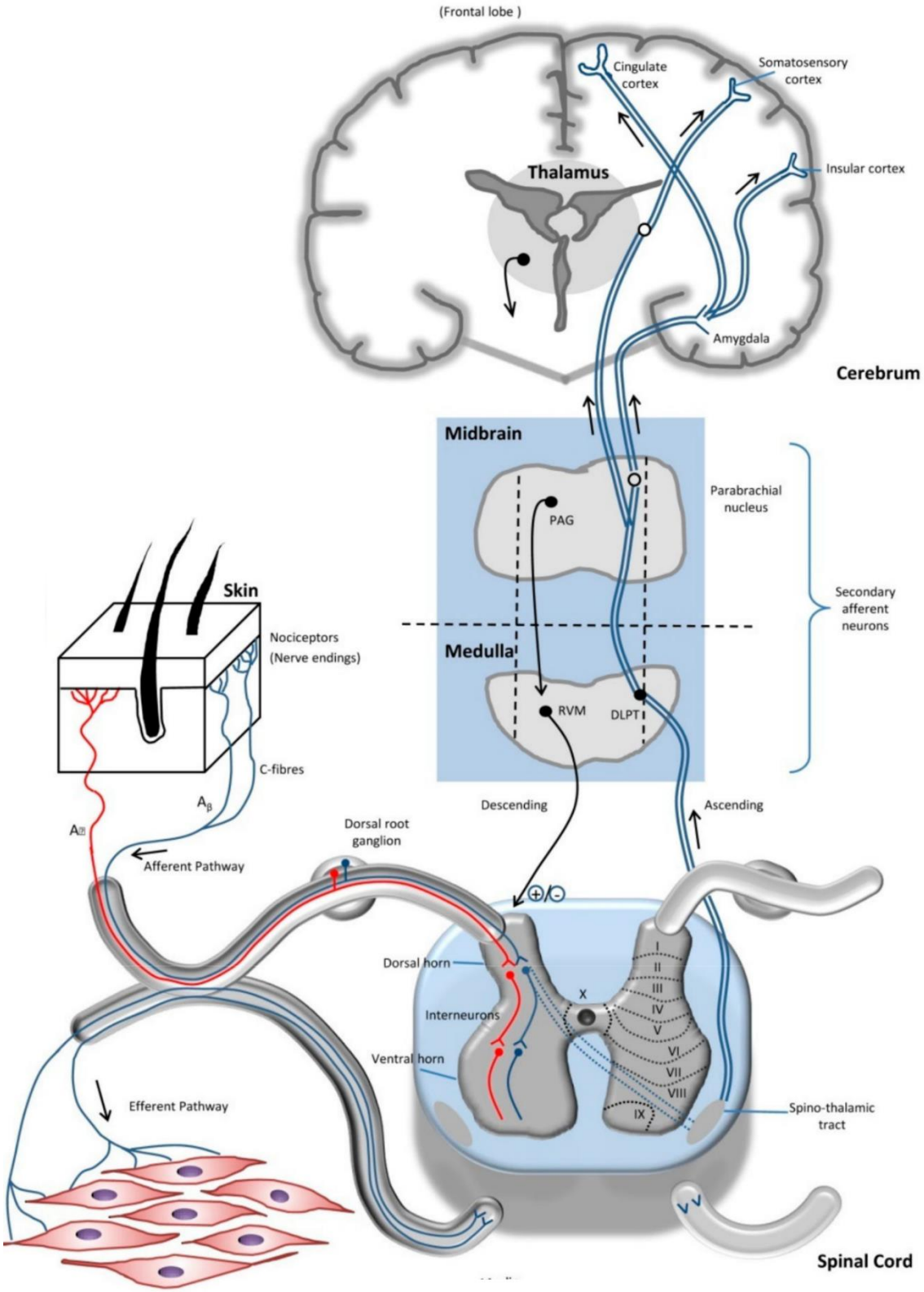


Figure 1. Route of pain transmission (Yam et al., 2018).

Typically, the lateral spinothalamic tract locating in the anterolateral region of spinal white matter conveys information of pain and temperature to the thalamus, while the

anterior spinothalamic tract within the anterior region of spinal white matter is responsible for carrying the sensory impulse of coarse touch and firm pressure towards the thalamus (Blanco et al., 2018, Almeida et al., 2004).

Of notice, the noxious stimuli from the face transmit through the trigeminal ganglion, ascend through the trigeminal spinal nucleus, and synapse in the thalamus, then terminate in the cortex. The spinal cord is not involved in the facial pain pathway (Rajneesh and Bolash, 2018). Besides, the cingulate cortex, insular cortex, and hippocampus participated in the formation of affective consequences associated with pain, such as depression and anxiety (Yalcin et al., 2014).

1.4 Mechanisms of neuropathic pain following SCI

During the last decades, many researches have provided an increasing knowledge about pain, but the mechanisms underlying SCI neuropathic pain are still not well understood. Unlike nociceptive pain, neuropathic pain resulting from a joint action of multiple pathological mechanisms serves no purpose for our body's defense system. There are several hypotheses trying to explain how neuropathic pain happens after SCI, and implied that neuronal hyperexcitability is the key point of the production of enhanced pain following spinal cord injury. Peripheral sensitization, intracellular hyperexcitability pathways in neurons, hypofunction of inhibitory system, neuro-immune interactions, and the plasticity of neurons may contribute to neuronal hyperexcitability evoking chronic neuropathic pain (Gwak and Hulsebosch, 2011b).

1.4.1 Peripheral sensitization

The peripheral sensitization mechanism underlying neuropathic pain after SCI is mainly about the increased excitability of DRG and the decreased pain thresholds. In the light of the recordings from peripheral nociceptors, the enhanced responses to evoked stimuli (allodynia, hyperalgesia) and increased spontaneous activity

(spontaneous pain) are observed in SCI rat models (Carlton et al., 2009). The immune system and the sympathetic nervous system play vital roles in the process of peripheral sensitization.

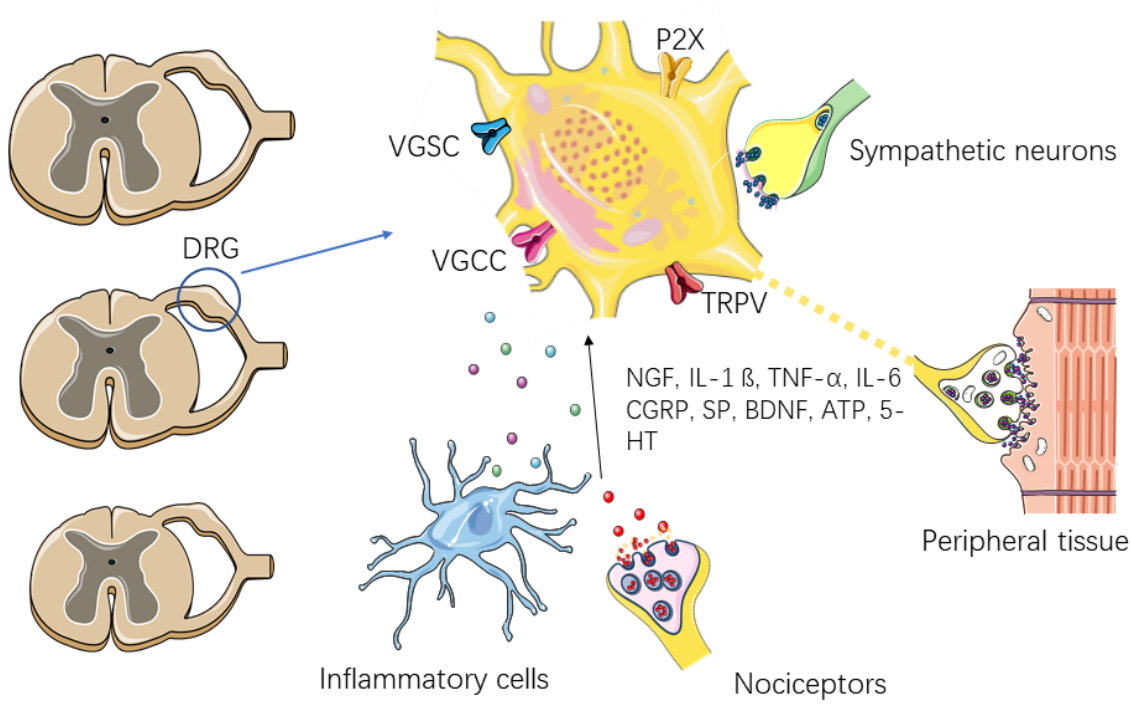


Figure 2. An overview of peripheral sensitization mechanisms (drawn with software Microsoft PowerPoint).

As a consequence of the inflammatory reaction, a massive amount of endogenous and exogenous substances is released from the nociceptors or inflammatory cells. Those substances, such as cytokines (IL-1, IL-6, TNF-α), calcitonin gene related peptide (CGRP), serotonin (5-HT), increase the sensitivity and excitability of primary afferent neurons locating in DRG areas (Kraychete et al., 2008, Sommer and Kress, 2004, Millan, 1999). In addition, the sympathetic axons sprout within the regions of DRG and form a synapse-similar functional structure interacting with the ganglion cell bodies. The newly established connections could upregulate the ectopic activity in the DRG which would facilitate the onset of neuropathic pain (Ramer and Bisby, 1997, Devor et al., 1992, Amir and Devor, 1993).

During the process of the inflammation and sympathetic sprouting, the modulation of ion channels primarily makes contribution to the occurrence, development and

persistence of neuropathic pain. The common pain regulation-related iron channels contain voltage-gated sodium channels (VGSCs) (Ma et al., 2019), voltage-gated calcium channels (VGCCs) (Sekiguchi et al., 2018, Leo et al., 2017), transient receptor potential cation channels (TRPV) (Levine and Alessandri-Haber, 2007, González-Ramírez et al., 2017), acid-sensing ion channels (ASICs) (Baron et al., 2018, Gründer and Pusch, 2015), ATP-gated P2X receptor channel (Chen et al., 2005, Gum et al., 2012).

1.4.2 Intracellular hyperexcitability pathways in neurons

In response to SCI, the enhanced ectopic firing produced from injured and adjacent fibers tremendously increases the levels of extracellular glutamate and accelerates the release of ROS and proinflammatory cytokines. Those reactive events activate a massive cations influx and further initiate the intracellular signaling pathways that ultimately promote the alteration of ion channels and their corresponding receptors on the membrane of neurons. This process amplifies the signals coming from primary afferents, eventually results in neuronal hyperexcitability and persistent neuropathic pain (Gwak et al., 2013, Crown et al., 2006, Gwak and Hulsebosch, 2012).

The intracellular signaling pathways mainly involve mitogen-activated protein kinase (MAPK) transcriptional pathways which can regulate the protein expression (Ji and Strichartz, 2004). The activation of N-methyl-D-aspartate receptor (NMDA receptor: an ionotropic glutamate receptor), voltage-gated calcium channels (VGCCs), TRPV, and the inotropic ATP receptor P2X3 trigger calcium influx and subsequently activate calcium-dependent protein kinases and enzymes, followed by the activation of MAPK pathways (Ma and Quirion, 2005, Ji and Strichartz, 2004). For instance, the initiation of the p38-MAPK pathway modulates neuronal hyperexcitability in the dorsal horn area. The intrathecal administration of an inhibitor (SB203580) can downregulate the level of the phosphorylated form of p38-MAPK to reduce the neuropathic pain caused by SCI (Crown et al., 2008).

1.4.3 Hypofunction of the inhibitory system

It is well documented that gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter throughout the CNS. Glutamic acid decarboxylase (GAD) can catalyze the decarboxylation of glutamate and convert it into GABA. The released GABA binds to specific transmembrane receptors which promote the outflow of potassium ions and the inflow of chloride ions, and finally accelerate the hyperpolarization at the pre- and post-synaptic neurons. Normally, the distribution and concentration of glutamate, GABA, and chloride ions are maintained at a dynamic balance. However, SCI will destroy the balance and lead to persistent neuronal hyperexcitability which can cause increased pain behaviors (Gwak and Hulsebosch, 2011a). In recent studies, it is suggested that there are different pathophysiological mechanisms inducing the hypofunction of the GABA inhibitory system after SCI.

Firstly, the initial mechanical and ischemic insults can cause cell death, and the secondary injury cascades in the lesion and the surrounding area lead to further cell death and cell apoptosis. In the moderate contusion injury mice models, it was observed that the number of GABA immunopositive neurons significantly decreased in the dorsal horn six weeks after the injury (Meisner et al., 2010).

Secondly, several prior studies suggest that in the SCI process, the occurrence of neuropathic pain is relevant to the decreased glutamic acid decarboxylase (GAD) level (Gwak and Hulsebosch, 2011a). As is well-known, GAD is a rate-limited enzyme and exists in two isoforms (GAD65 and GAD67) which are responsible for the synthesis of GABA. Therefore, the downregulation of GAD expression is considered to cause the reduced production of GABA (Cordero-Erausquin et al., 2016). In the chronic constriction injury (CCI) and spared nerve injury (SNI) models, the results of immunohistochemistry and western blot showed the decreased level of GABA, as well as the downregulated expression of GAD65 and GAD67 on the ipsilateral side (Moore et al., 2002, Meisner et al., 2010). Gwak and colleagues found that in the hemisection rat models, the intrathecal administration of propentofylline can attenuate allodynia, decrease glial activation, and preserve the function of GABA

inhibitory system by modulating the expression of GAD (Gwak et al., 2008).

Thirdly, the overproduction of GABA transporters (GATs) is significant for the downregulation of extracellular GABA concentration. GABA transporters are widely distributed on the plasma membrane on the cells of the CNS and they can transiently translocate the extracellular GABA to facilitate the recycle of GABA (Scimemi, 2014). Under the pathological circumstances, the activation of target intracellular signal transduction pathways increases the GATs cell membrane expression that promotes the GABA uptake and contributes to the hypofunction of GABAergic neurons, and then trigger neural hyperexcitability in the site of the dorsal horn. For instance, Masocha's team found that the GAT inhibitors could attenuate the pain development and limit the maintenance of neuropathic pain (Fijałkowski et al., 2017, Masocha and Parvathy, 2016).

Lastly, the abnormal activation of cation-chloride cotransporters (CCCs) may lead to decreased GABA inhibitory regulation. The CCCs include Na^+-K^+-Cl^- cotransporter 1 (NKCC1) and the K^+-Cl^- cotransporter 2 (KCC2). The NKCC1 is responsible for transporting Cl^- into the cells while the KCC2 focuses on moving Cl^- to the extracellular space (Blaesse et al., 2009). Given that $GABA_A$ receptors are primarily chloride-permeable channels, CCCs regulate GABA signaling via generating transmembrane chloride gradients and mediating the elevated neuronal excitability (Blaesse et al., 2009, Otsu et al., 2020, Hasbargen et al., 2010).

The understanding of the GABA inhibitory system brings about a conception that enhancing the function of GABAergic neurons may be a promising therapeutic strategy to alleviate intractable neuropathic pain after SCI (Gwak et al., 2006, Jergova et al., 2019).

1.4.4 Neuro-immune interactions in the spinal cord

As mentioned above, the pathophysiological changes of SCI generally go through three periods in sequence: the acute phase, secondary phase, and chronic injury phase. During these processes, the interactions between the nervous system and the immune

system lead to a series of inflammatory responses in the lesion, which might be a common mechanism underlying pain hypersensitivity. Glial cells (especially the microglial cells) are believed to play prominent roles in this progress (Vallejo et al., 2010, Mika et al., 2013).

In normal conditions, microglia are classified as resting macrophages, comprising 5% to 10% of the glial population (Filiano et al., 2015). When suffering injuries, ischemia or infection, microglia, as the professional phagocytes, act as the first and main form of active immune defense in the CNS (Watkins L.R., 2007, von Bartheld et al., 2016, Allen and Lyons, 2018). In the case of SCI, the microglia are activated within minutes and remain active for weeks. Activated microglia exhibit morphological hypertrophy and strong proliferation, release inflammatory substances such as IL-1, TNFα, IL-6, and chemokines, and thereby regulate pain-related ion channels and activate intracellular downstream cascades, and ultimately increase neuronal hyperexcitability (Gwak et al., 2017). For instance, the inhibition of NADPH oxidase (NOX2) signaling significantly reduced microglia infiltration via regulating the IL-10/miR-155 pathway, alleviating post-SCI pain behavior, and improving motor function in contusion mice models (Sabirzhanov et al., 2019).

1.4.5 The plasticity of neurons

Aberrant afferent plasticity is another driving force for persistent neuropathic pain after SCI (Farrell et al., 2019). On the one hand, the sprouting of the primary afferent fibers and the reorganization of synaptic circuits permanently change the neuroanatomy of the dorsal horn and induce the neuronal hyperexcitability. Under normal physiological conditions, pain-conducting primary afferent fibers (Aδ and C fibers) transport impulse and predominantly terminate in the superficial dorsal horn (laminae I–II) (Farrell et al., 2019, Yam et al., 2018). In the process of SCI, the secretion of neurotrophic factors stimulates the regrowth of axons, and those sprouting fibers expand their terminal areas into the deep region of the dorsal horn

(laminae III– V) and reorganize new synaptic circuits (Farrell et al., 2019, Yam et al., 2018). The aberrant distribution of pain-conducting primary afferents contributes to persistent neuronal hyperexcitability (Gwak and Hulsebosch, 2011b). On the other hand, during SCI, the non-nociceptive primary afferent fibers transform into nociceptive fibers via molecular phenotype switching, leading to central sensitization (Farrell et al., 2019).

Collectively, neuronal hyperexcitability caused by different mechanisms plays a key role in the production, spread and persistence of the SCI-induced neuropathic pain.

1.5 Aim of this study

My colleague, Tianci Cheng, has successfully established a spinal cord hemisection rat model for studying SCI-induced neuropathic pain in previous work (Cheng, 2019). My part in this project was the histologic and histochemical evaluation and comparison of the different tissue samples. The goals of this research were listed as follows:

- Establish a method to identify and harvest the transplanted tissues and prepare them for histologic and immunohistochemical evaluation.
- Use different histologic and immunochemical methods to identify different cell types in the relevant tissue.
- Compare the different animal groups regarding the condition of chronic neuropathic pain and the corresponding location and survival of pain relevant cell types.
- Evaluate, whether the transplantation of GABAergic neural stem cells could relieve the SCI-induced neuropathic pain.

2. Materials and Methods

2.1. Animal experiment

2.1.1 Animals

Forty-five Sprague Dawley adult male rats (weight 240g-260g) were used in this experiment. They were housed in standard rat cages with available food and water. During the trial period, the average room temperature was 20-23℃ and room humidity was about 50%, and the dark/light cycle is 12 hours.

All animal care, handling, and surgical procedures in this study were approved by the Ethic Committee for animal research in the state of Baden-Württemberg (protocol number: C5/12). (Cheng, 2019)

2.1.2 Experimental design

Forty-five Sprague Dawley male rats in this project were randomly divided into three groups (i.e. Group A, Group B and Group C), receiving different experimental interventions (see Figure 3). 15 rats in group A went through sham operations, while on the other rats in groups B and C were performed lateral dorsal hemisection surgery at T13 level in the spinal cord with 1mm cutting depth. Within the first week of the post-surgical period, vital signs, motor functions and pain properties were assessed every day. Then, the rats of these three groups went through the second surgeries. Rats in group A were processed with sham operations again, while those in groups B and C were transplanted with PBS (Phosphate-buffered saline) and GABAergic neural stem cells, respectively. Group A: sham operation group. Group B: transplanted with PBS ("SCI" group). Group C: transplanted with GABAergic neural stem cells ("SCI + GNSCT" group). Vital signs, motor functions and pain properties were also measured every day in the first week of the post-operational period and then once a week until

the 8th week. (Cheng, 2019)

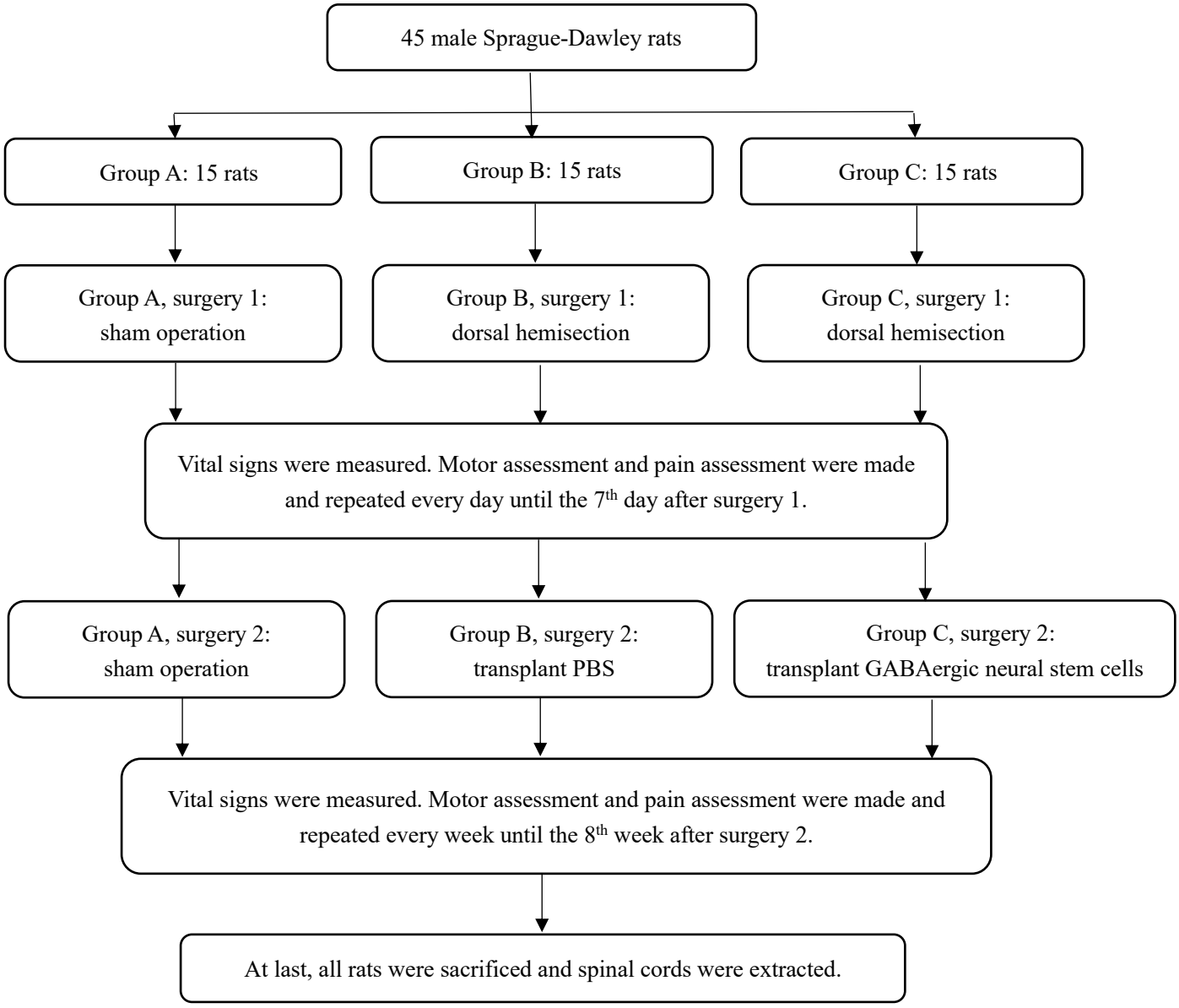


Figure 3. Flow chart illustrating animal experiments.

2.1.3 Surgical procedure of dorsal hemisection

Before surgery, vital signs of rats were measured and motor assessment and pain assessment were made as well. The rats were deeply anesthetized by intraperitoneal injection of a mixture of ketamine (Ketaset, 7-10mg/100g bodyweight; Parke Davis, Germany) and xylazine (Rompun 1mg/100g bodyweight; Bayer, Germany). Each

animal was kept on a 37±0.5°C heating pad prior to surgery until it recovers from the anesthesia to maintain the body temperature. The rat's back was all shaved and aseptically prepared before the surgery. The rat's vertebrae were fixed with the stereotactic apparatus. A longitudinal skin incision was made and the paraspinal musculature was bluntly dissected to the spinous processes. Thus, the laminae were exposed. A laminectomy was conducted at vertebral level T12 or T13. As shown in Figure 3, animals in the sham operation group received a laminectomy without inducing injury to the dorsal horn while animals in the other two groups received dorsal horn hemisection. A No. 11 scalpel blade was used to hemisect the spinal cord from cranial to the dorsal root entry zone with 1 mm depth of cut (see Figure 4). Then, the muscles, fascia and skin were sutured in order. (Cheng, 2019)

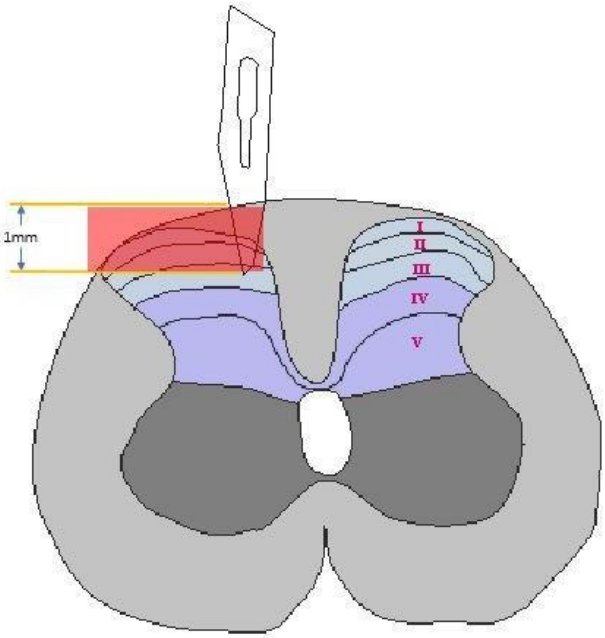


Figure 4. T13 spinal cord hemisection (Cheng, 2019).

2.1.4 Transplantation of GABAergic neural stem cells

One week after the first surgery, rats in group A were processed with sham operations again, while those in groups B and C were transplanted with PBS and GABAergic neural stem cells, respectively. Under aseptic conditions, these animals were

anesthetized. After the T13–L1 vertebrae had been identified by palpating the last rib, a laminectomy was applied to expose L3–L4 lumbar spinal cord. GABAergic neural stem cells were loaded into the Hamilton microinjection syringe and injected into the damaged side. The syringe was placed 0.5 mm from the dorsal central vein and an injection was given at depth of 0.5 mm to 1 mm from the dorsal lumbar spinal surface. A volume of 4µl GABAergic neural stem cells (5×10^4 cells/µl) was injected to the rats in group C at a rate of 1µl/min, while 4µl PBS was injected in rats in group B. Later, muscles were sutured in layers and the skin was closed at last. The rats were then placed to heated cages for recovery and received cyclosporine A from one day before transplantation until being sacrificed. (Cheng, 2019)

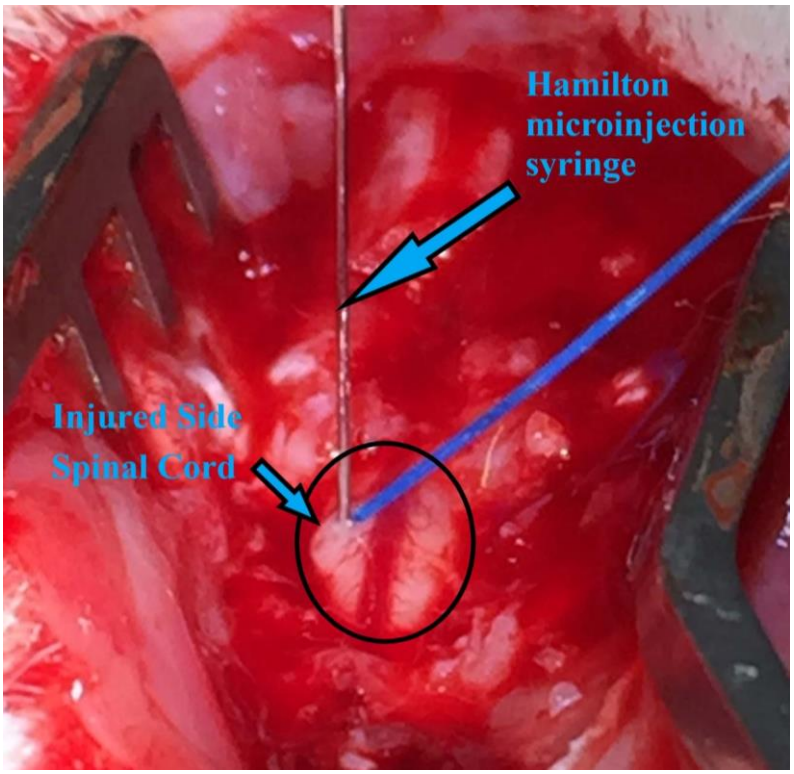


Figure 5. Transplantation of cells or PBS (Cheng, 2019).

The GABAergic neural stem cells were isolated and differentiated by Tianci Cheng from embryonic brain tissues in embryonic day 14 Sprague Dawley rats. The methods about the isolation and differentiation of GABAergic neural stem cells can be seen in Tianci Cheng's MD thesis "GABAergic neural stem cells transplantation after spinal cord injury induced chronic neuropathic pain in a rat model" (Cheng, 2019), from page 22 to 26.

2.1.5 Motor function semiquantitative assessment

The Basso, Beattie and Bresnahan (BBB) locomotor rating scale is originally developed for evaluating the behavioral consequences of thoracolumbar SCI to the rat (Basso et al., 1995), which has been modified and widely used in other species including mice, opossums and dogs (Basso et al., 2006, Wang et al., 1998, Song et al., 2016). This BBB scale is a highly sensitive and repeatable indicator to assess functional status，and the values of this scale range from 0 to 21 points. Rats were placed in a round plastic pool (100-cm diameter,17-cm wall height) and their activities' videos were recorded for monitoring the motor function (Figure 6). Two trained and blinded observers assessed 90 hindlimbs of 45 rats independently from the day before surgery to the 9th week after spinal cord injury. (Cheng, 2019)

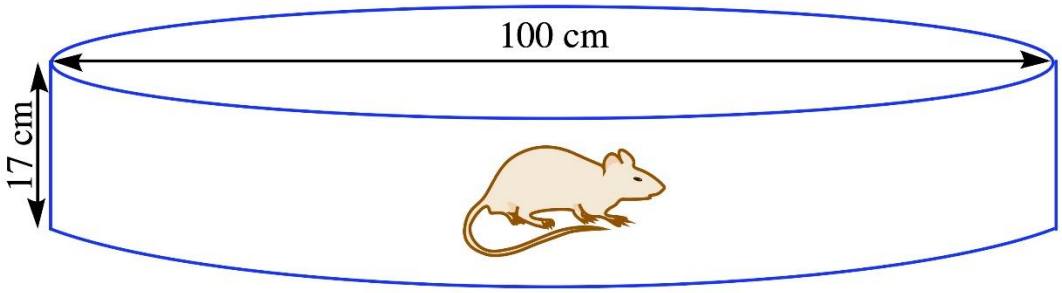


Figure 6. Scheme of BBB locomotor rating scale testing.

The BBB scale categorizes combinations of limb movement, trunk position and stability, abdomen, paw placement, stepping, coordination, toe clearance, predominant paw position, trunk instability and tail, measuring motor recovery of the rat after SCI. A score of 0 suggests no observable hindlimb movement, while a score of 21 indicates full recovery of the rat's motor function. (see Table 3)

BBB LOCOMOTOR RATING SHEET

Rat#:_____ Date:____/___/___ DPO:_____ Score:L____R____

Limb Movement			Trunk Position		Abdomen	Paw Placement			Stepping		Coordination	Toe Clear.	Predominant Paw Position		Trunk Instability	Tail
Hip	Knee	Ankle	Side	Supp.		Sweep	Plantar PL.		Weight	1st. Toe			Initial Contact	Lift off		
L R	L R	L R					W/O Supp.	W Supp.	L R	L R	L R	L R	L R	L R		
Ø Ø	Ø Ø	Ø Ø	L R	Yes	Drag	L R	L R	L R	Ø Ø	Ø Ø	Ø Ø	Ø Ø	I I	I I	Yes	Up
S S	S S	S S			Parallel				O O	O O	O O	O O	E E	E E		
E E	E E	E E	Mid	No	High				F F	F F	F F	F F	P P	P P	No	Down
									C C	C C	C C	C C				

Comments:

Ø - No movement
S - Slight Movement
E - Extensive movement

Ø - Never 0% Clearance <=5%
O - Occasional <=50%
F - Frequent 51-94%
C - Consistent 95-100%

I - Internal Rotation
E- External Rotation
P - Parallel

Table 3. BBB locomotor rating sheet (Basso et al., 1995).

2.1.6 Pain assessment

In this experiment, an electronic von Frey apparatus (Dynamic Plantar Aesthesiometer, Cat. No. 37400-001, Ugo Basile, Italy) was used to evaluate static mechanical pain hypersensitivity in animals. This device consists of a movable touch-stimulator unit, a microprocessor-controlled electronic unit, a perforated metal platform and a modular animal enclosure (Figure 7). In order to reduce bias, all rats were placed in the animal enclosure for a period every day one week before surgery. Besides, they were put into the enclosure for about 20 minutes to be acclimatized to the cages before the test. After zeroing the read-out, the tip of the force transducer was positioned to the footpad center. Then, the force of the touch-stimulator was increased gradually and linearly until a clear hind paw withdrawal reflex is observed. Meanwhile, the pressure value that elicited the response was noted. Every hind paw was tested five times to calculate the mean force and mechanical stimulations applied on the same hind paw ought to be at least 3 min apart. (Cheng, 2019)

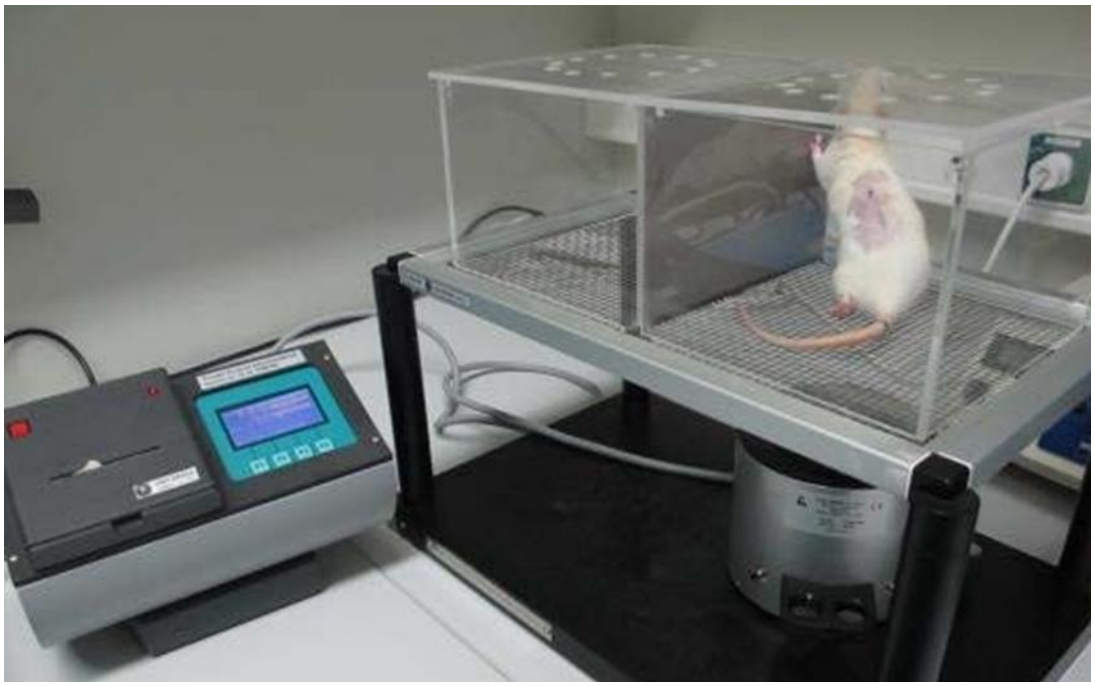


Figure 7. Apparatus for the electronic von Frey test (Cheng, 2019).

2.2 Tissue staining

2.2.1 Tissue preparation

All rats were sacrificed by injecting lethal intraperitoneal ketamine (Ketaset, Parke Davis, Germany) eight weeks after the second surgery. The Spinal cords were perfused with PBS (liquid temperature: 4℃) intracardially, followed by 4% paraformaldehyde in 0.1 M PBS. After that, spinal cords were immediately removed and post-fixed in the fixative at a temperature of 4℃ for 3-4 days. Slices of 5 mm thickness of the injured portion were cut (Figure 8) to prepare spinal cord tissue blocks. The segments at the T13 level of the spinal cords in the sham operation group were also cut to create blocks.

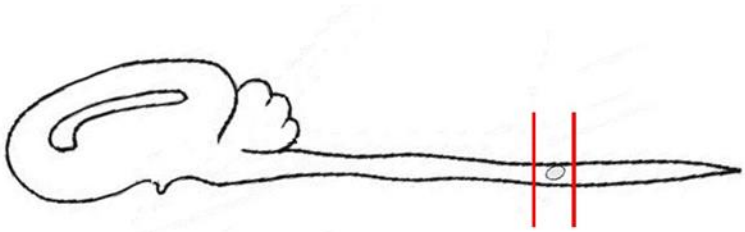


Figure 8. The injured segment was cut.

2.2.2 Paraffin embedding

In 1869, paraffin wax was applied as an embedding medium to histological study. Since then, paraffin embedding has been extensively used for infiltrating and supporting tissue blocks. The embedded tissue can be kept stable for many years. In this project, an automated tissue processor was used to process tissue and produce paraffin blocks. The experimental protocol of paraffin embedding is described below (Figure 9). Then, we embedded the spinal cord blocks for further sectioning and staining (Figure 10).

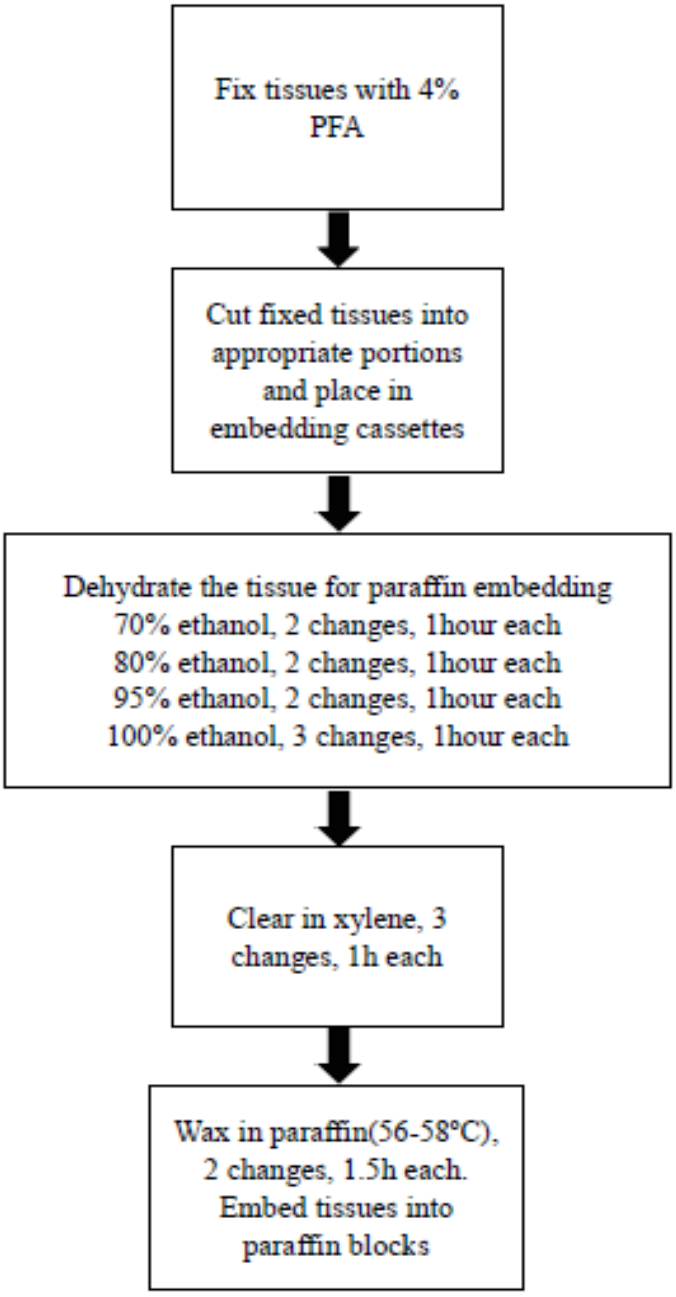


Figure 9. Scheme of the Paraffin embedding protocol.

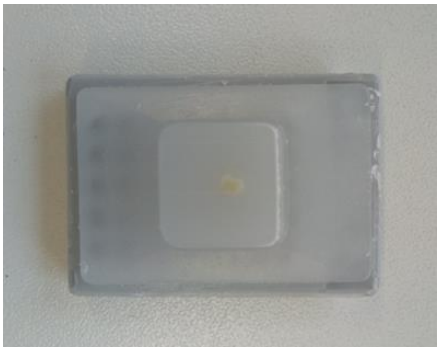


Figure 10. Paraffin-embedded spinal cord tissue block.

2.2.3 Sections cutting and mounting

The paraffin blocks were trimmed to an optimal cutting surface, and they were cut into 3 μm slices. Then, the paraffin slices were moved in the 40-45ºC water bath to remove possible wrinkles. Glass slides were used to retrieve and position those swimming paraffin sections. Afterwards, the sections were dried at 37ºC and mounted on the glass slides.

Figure 11. Slices of the sectioned spinal cord.

2.2.4 Immunohistochemistry staining

Immunohistochemistry is a useful method to visualize specific cellular components by applying labeled antibodies to bind target antigens in situ. There are numerous approaches in immunohistochemistry methodology, while in this project, DAB (3,3′ -Diaminobenzidine) staining was utilized to detect target antigens in the sectioned spinal cord. The scheme of the immunohistochemistry protocol is shown in Figure 12.

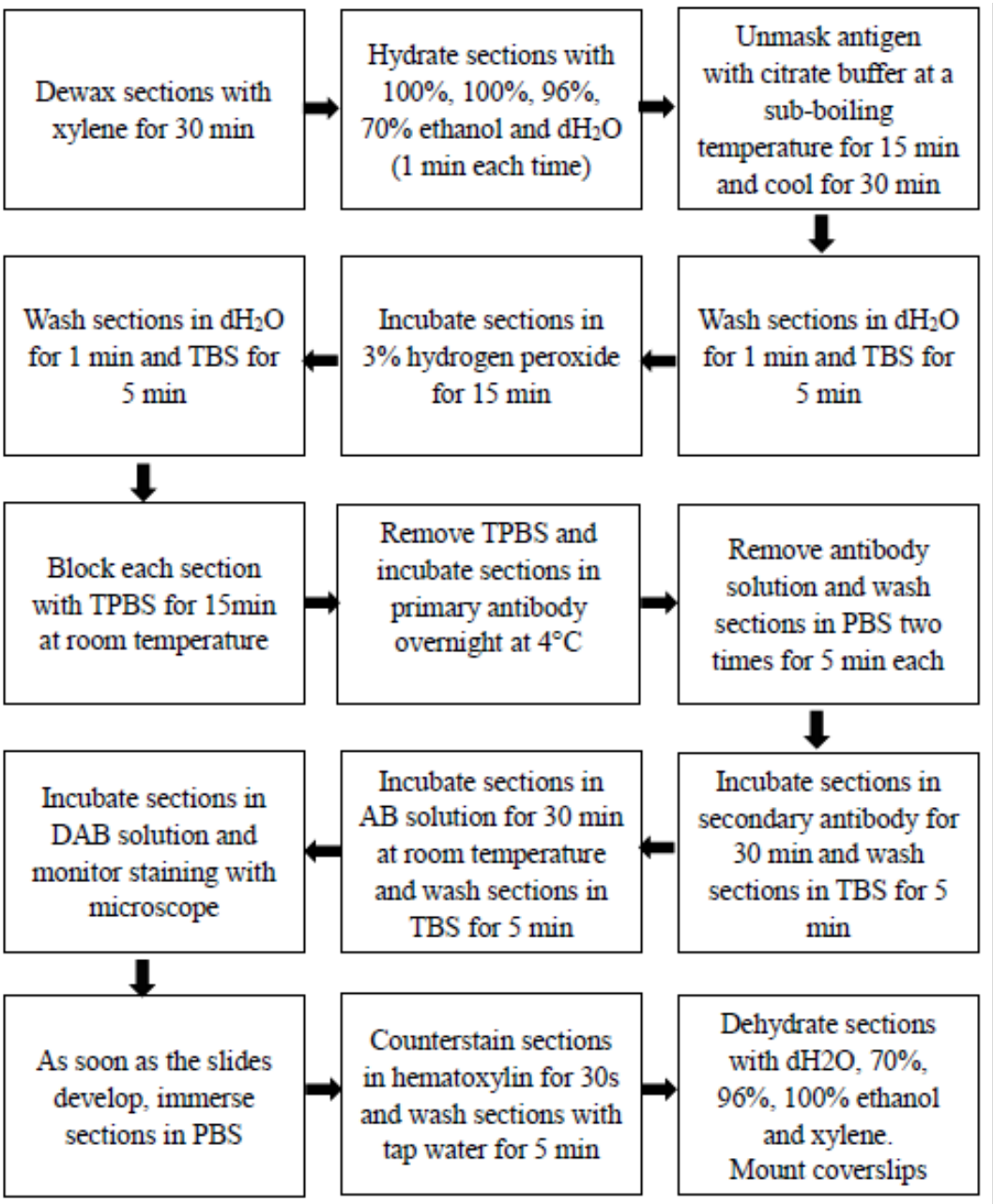


Figure 12. Scheme of the immunohistochemistry protocol. (Note: dH_2O = distilled water; TBS = Tris-buffered saline; TPBS = Tris phosphate buffered saline; AB = avidin biotin)

The antibodies used in this study was listed as follows:

monoclonal anti-NeuN produced in mouse (Abcam, ab104224),

polyclonal anti-γ-Aminobutyric acid (GABA) produced in rabbit (Sigma-Aldrich, A2052),

polyclonal glutamic acid decarboxylase (GAD) produced in rabbit (Sigma-Aldrich, SAB4501075),

recombinant Anti-Iba1 antibody produced in rabbit (Abcam, ab178846),
polyclonal anti-glial fibrillary acidic protein (GFAP) (Sigma-Aldrich, G9269),
secondary antibody fragment (DAKO, Hamburg, Germany).
avidin biotin complex (Vector Laboratories, PK-4000)

2.3 Evaluation and statistical analysis

2.3.1 Image analysis

After staining, the spinal cord sections were photographed and examined by light microscopy (Nikon Cool scope, Nikon, Düsseldorf, Germany). Two independent and blind observers analyzed those captured images with Image-Pro Plus software (Image-Pro Plus 6.0, Media Cybernetics Corporation, USA). The NeuN, Ionized calcium-binding adapter molecule 1(Iba-1) and GFAP positive cells in spinal cords were manually counted.

2.3.2 Statistical analysis

All data in this study were calculated and shown as mean ± standard deviations (SD). The data were analyzed with the Graph Pad Prism 7.0 software (Graph-Pad Software Inc., San Diego, CA, USA), and a difference of P values <0.05 was defined as statistically significant. One-way Analysis of Variance (ANOVA) was conducted to compare the differences of behavioral events or immunohistochemistry intensity between the sham operation group, the “SCI” group and the “SCI+GNSTC” group. Correlations between immunohistochemistry intensity of GABA in superficial dorsal horn and motor function assessed with a BBB scale were analyzed with the Pearson correlation analysis.

3. Results

3.1 Results of motor function assessment in rats

Table 4. BBB Scores quantification (Cheng, 2019).

	BBB score (Mean ± SD)		
	Sham Operation group	Transplant PBS Group	Transplant Cells Group
Pre SCI	21.000±0.000	21.000±0.000	21.000±0.000
D1 after SCI	19.533±1.187	10.867±2.560	12.267±2.052
D2 after SCI	20.733±0.458	10.800±2.145	12.600±1.805
D3 after SCI	21.000±0.000	12.400±2.324	13.933±2.251
D4 after SCI	21.000±0.000	13.867±2.532	15.467±2.446
D5 after SCI	21.000±0.000	15.600±2.823	16.933±2.434
D6 after SCI	21.000±0.000	17.667±2.690	18.400±2.558
D7 after SCI	21.000±0.000	18.667±2.610	19.867±1.959
D1 after Trans.	19.733±1.280	14.867±3.441	16.333±2.093
D2 after Trans.	20.533±0.743	15.333±3.039	17.200±2.178
D3 after Trans.	20.933±0.258	16.667±3.309	18.667±2.469
D4 after Trans.	21.000±0.000	18.067±2.815	19.867±2.031
D5 after Trans.	21.000±0.000	19.133±2.416	20.400±1.549
D6 after Trans.	21.000±0.000	19.600±1.844	20.733±0.799
D7 after Trans.	21.000±0.000	20.400±1.056	20.867±0.516
W2 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W3 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W4 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W5 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W6 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W7 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000
W8 after Trans.	21.000±0.000	21.000±0.000	21.000±0.000

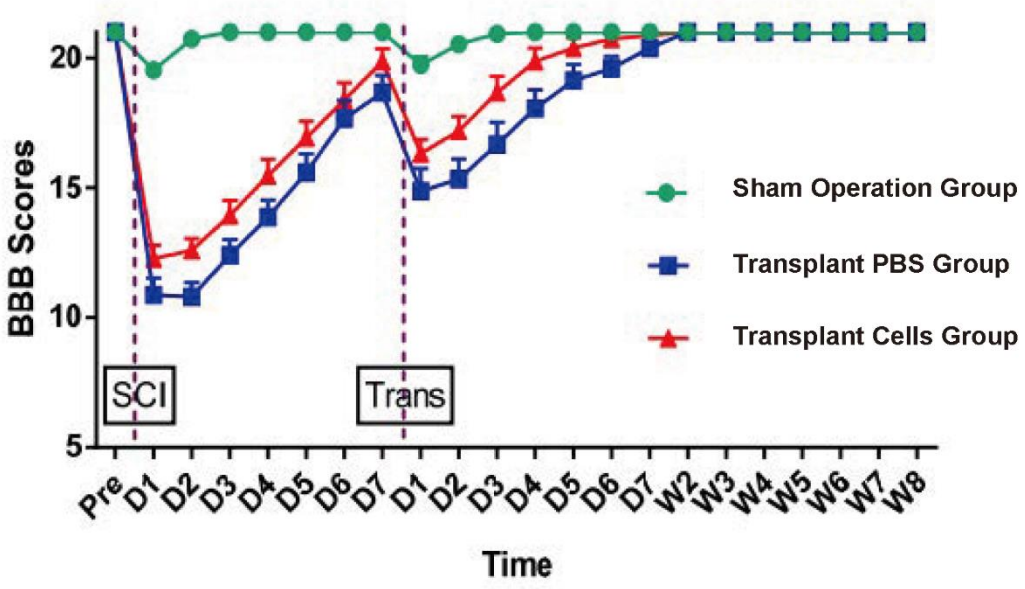


Figure 13. BBB Scores on interventional sides of the hindlimbs in rats coming from the sham operation group, PBS transplantation group and GABAergic neural stem cell transplantation group (Cheng, 2019). The blue line represents the sham operation group, the transplant PBS group is described with a green line, and the red line refers to the transplant cells group. The X-axis indicates the time from the day prior to spinal cord injury to the 8th week after transplantation. The Y-axis represents the motor function assessed with a BBB scale (Mean ± SD). The motor function was measured daily within the first week after SCI, then recorded daily in the first week after the transplantation surgery and later assessed weekly until the 8th week. "Pre" indicates before surgery. (Cheng, 2019)

3.2 Results of pain assessment in rats

Table 5. Pain assessment quantification (Cheng, 2019).

	Sham Operation Group	Transplant PBS Group	Transplant Cells Group
Baseline	50.000 ± 0.000	50.000 ± 0.000	50.000 ± 0.000
1W after SCI	45.829 ± 2.006	32.578 ± 2.700	28.357 ± 1.307
1W after Trans	45.764 ± 1.421	31.322 ± 2.350	28.621 ± 1.897
2W after Trans	45.414 ± 1.105	31.122 ± 2.509	30.971 ± 1.882
3W after Trans	45.500 ± 1.269	32.000 ± 2.221	32.014 ± 1.290
4W after Trans	45.464 ± 1.137	30.922 ± 1.490	33.321 ± 1.418
5W after Trans	45.607 ± 1.207	30.589 ± 1.574	33.743 ± 1.632
6W after Trans	45.450 ± 1.219	30.400 ± 2.513	34.450 ± 1.562
7W after Trans	45.486 ± 0.949	31.404 ± 1.590	34.721 ± 1.876
8W after Trans	45.807 ± 1.230	30.889 ± 1.456	34.550 ± 1.691

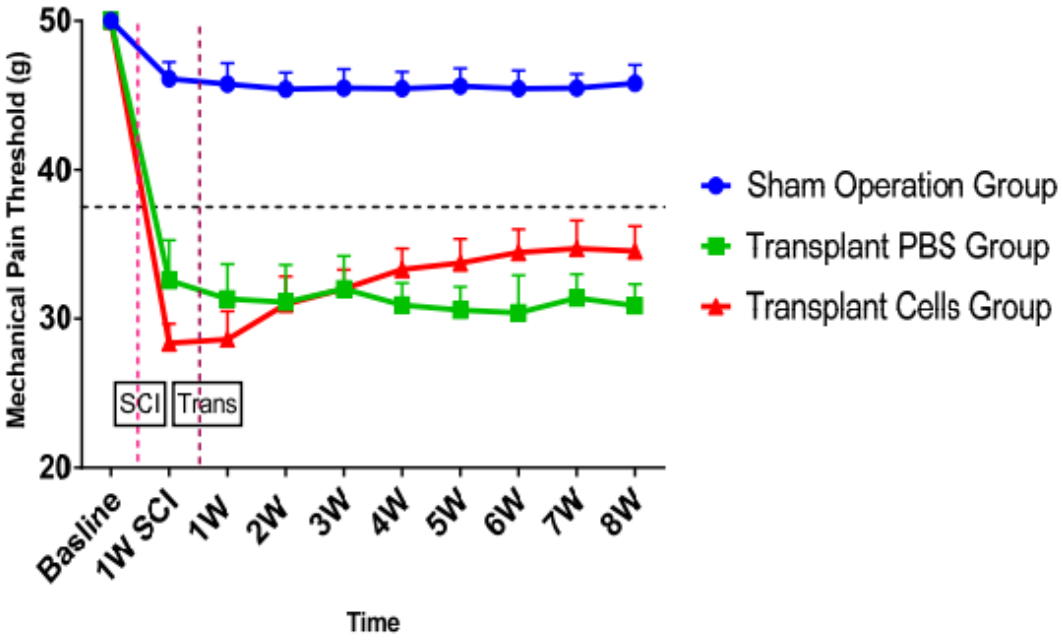
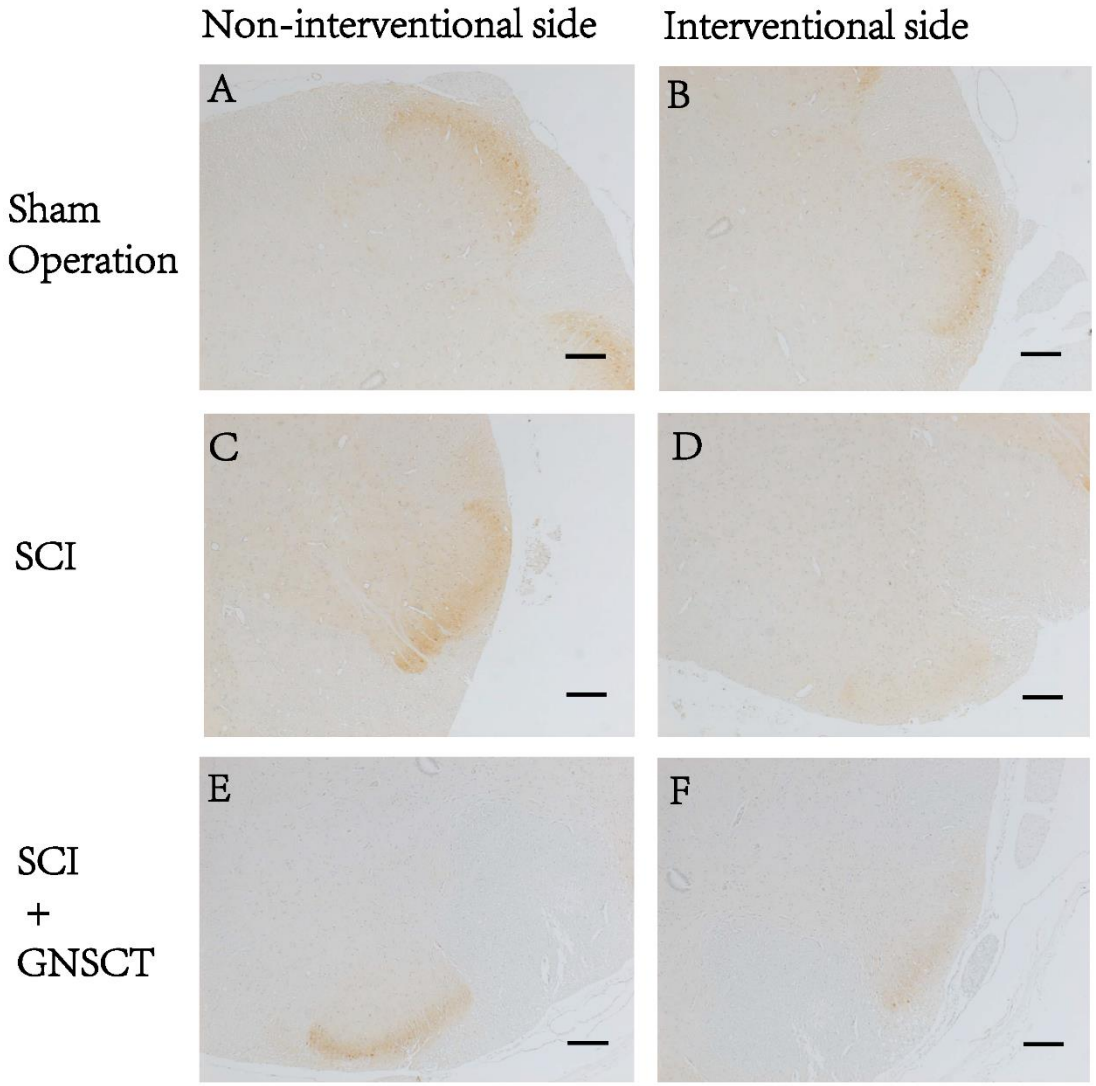


Figure 14. Changes in mechanical pain thresholds following dorsal horn hemisection in rats (Cheng, 2019). The blue line represents the sham operation group, the transplant PBS group is described with a green line, and the red line refers to the transplant cells group. The X-axis indicates the time from the day prior to spinal cord injury to the 8th week after transplantation. While the Y-axis represents the mechanical pain threshold (g) (Mean ± SD).

Within one week after receiving the first surgical intervention, the values of rats'pain threshold in the sham-operated group decreased slightly, while the pain threshold of the other two groups receiving dorsal hemisection surgery decreased significantly. After the second intervention, the pain threshold values of the sham-operated group were basically maintained at around 45.5, while the values of rats'pain threshold in the PBS-transplantated group were maintained at around 30.5. Moreover, the pain threshold of the cell transplantation group recovered to about 34.5 eight weeks after stem cell transplantation. (Cheng, 2019)

3.3 Results of the staining analysis

3.3.1 GABA staining results



*SCI: spinal cord injury

#GNSCT: GABAergic neural stem cell transplantation

Figure 15. Photomicrograph shows GABA level in the dorsal horns of the rats after the second surgery.

A. Photomicrograph of the non-interventional side of the group of sham operation;

B. Photomicrograph of the interventional side of the group of sham operation;

C. Photomicrograph of the non-interventional side of the group of "SCI";

D. Photomicrograph of the interventional side of the group of “SCI”;

E. Photomicrograph of the non-interventional side of the group of “SCI + GNSCT”;

F. Photomicrograph of the interventional side of the group of “SCI + GNSCT”.

Scale bar, 200 μm.

As shown in Figure 15, in the group of “SCI ” and the group of “SCI + GNSCT”, there is an obvious reduction of GABA in the ipsilateral at the injured level when compared with the contralateral dorsal horn.

GABA immunoreactivities in the selected area of dorsal horns were quantified by using image analysis to get semi-quantitative values and analyzed the data with Graphpad software (Figure 16).

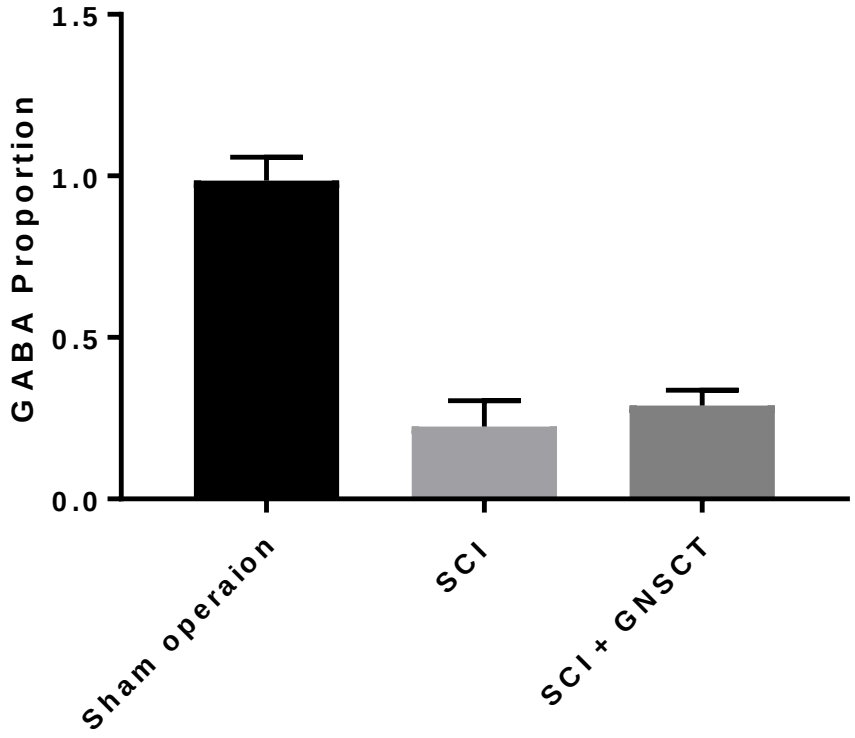


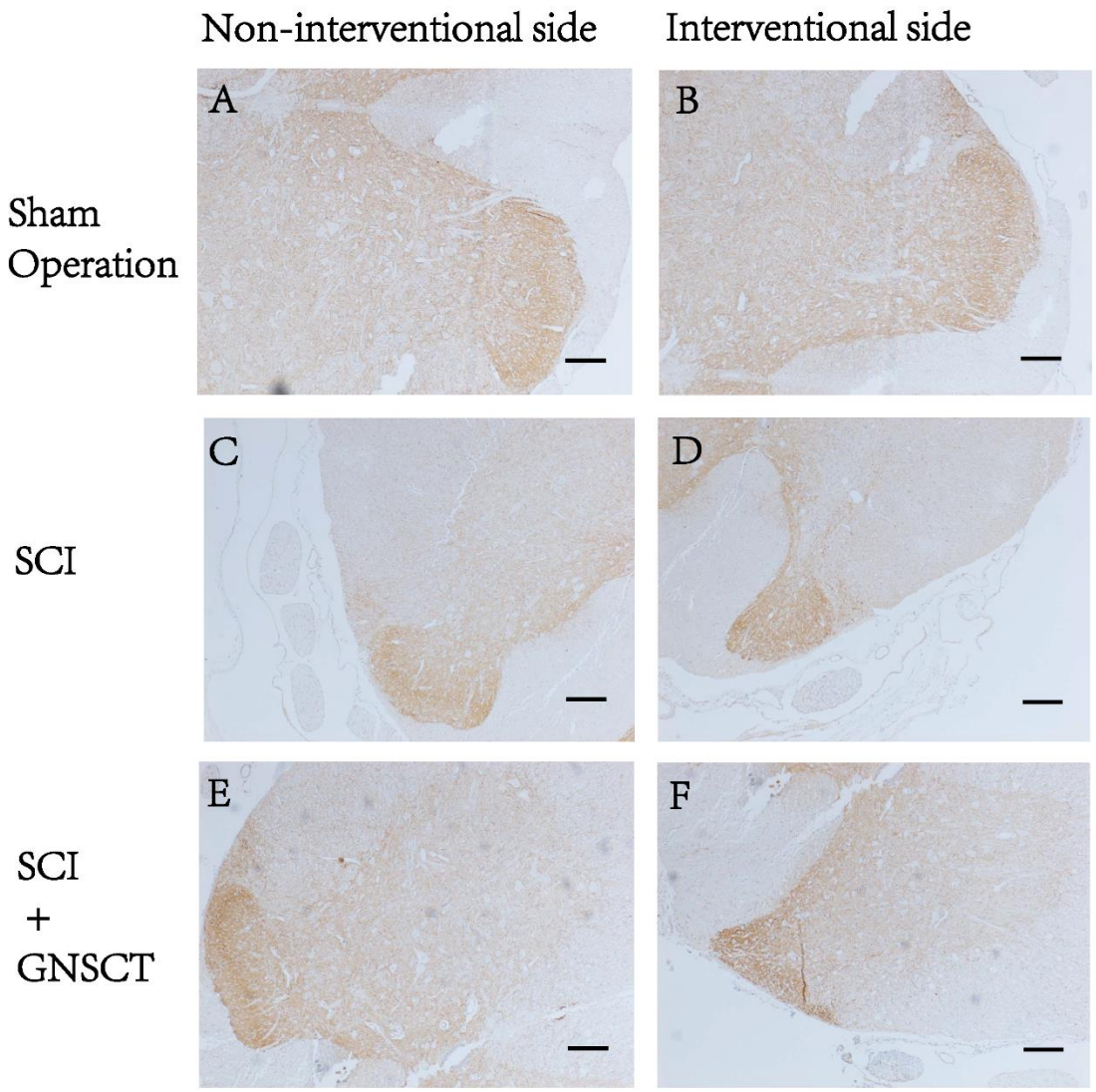
Figure 16. The column bar graph of the GABA proportion in the sham operation group, “SCI” group and “SCI + GNSCT” group.

GABA_Score = the integrated option density of GABA in the area of interest

GABA Proportion = GABA Score of the interventional side / GABA Score of the non-interventional side

As stated in the Figure 16, the GABA Proportion of the group “sham operation” was notably higher than both the group of “SCI” ($P < 0.0001$) and “SCI + GNSCT” ($P < 0.0001$). Compared with the group of “SCI”, the group “SCI + GNSCT” occupied a higher GABA Proportion ($P < 0.05$).

3.3.2 GAD staining results



*SCI: spinal cord injury

#GNSCT: GABAergic neural stem cell transplantation

Figure 17. Photomicrograph shows GAD in the dorsal horn.

A. Photomicrograph of the non-interventional side of the group of sham operation;

B. Photomicrograph of the interventional side of the group of sham operation;

C. Photomicrograph of the non-interventional side of the group of "SCI";

D.Photomicrograph of the interventional side of the group of "SCI";

E. Photomicrograph of the non-interventional side of the group of "SCI + GNSCT";

F. Photomicrograph of the interventional side of the group of "SCI + GNSCT"

Scale bar, 200 μm.

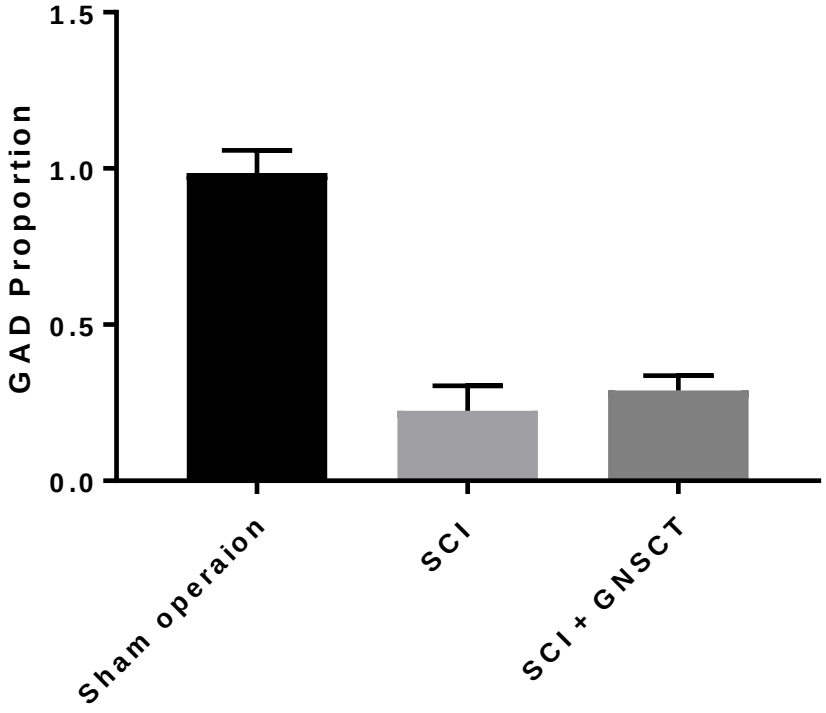


Figure 18. The column bar graph of GAD proportion in the sham operation group, "SCI" group and "SCI + GNSCT" group.

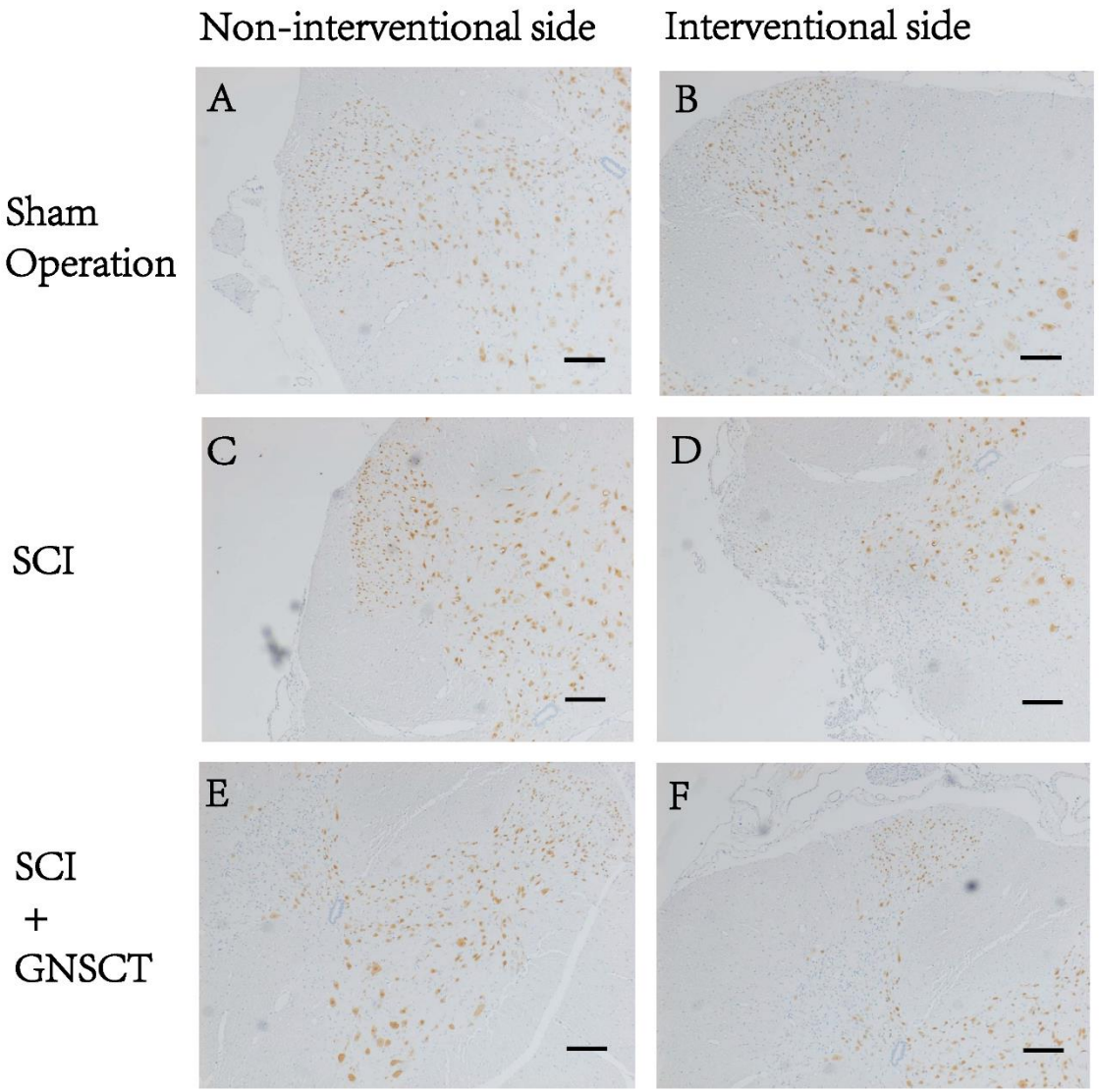
GAD_Score = the integrated option density of GAD in the area of interest

GAD Proportion = GAD Score of the interventional side / GAD Score of the non-interventional side

As stated in the Figure 18, the GAD Proportion of the group "sham operation" was obviously higher than both the group of "SCI" ($P < 0.0001$) and "SCI + GNSCT" (P

<0.0001). By comparison, the group "SCI + GNSCT" occupied a higher GAD Proportion than the group of "SCI" (P <0.05).

3.3.3 NeuN staining results



*SCI: spinal cord injury

#GNSCT: GABAergic neural stem cell transplantation

Figure 19. Photomicrograph shows NeuN positive neurons in the dorsal horn.

A. Photomicrograph of the non-interventional side of the group of sham operation;

B. Photomicrograph of the interventional side of the group of sham operation;

C. Photomicrograph of the non-interventional side of the group of "SCI";

D. Photomicrograph of the interventional side of the group of “SCI”;

E. Photomicrograph of the non-interventional side of the group of “SCI + GNSCT”;

F. Photomicrograph of the interventional side of the group of “SCI + GNSCT”

Scale bar, 200 μm.

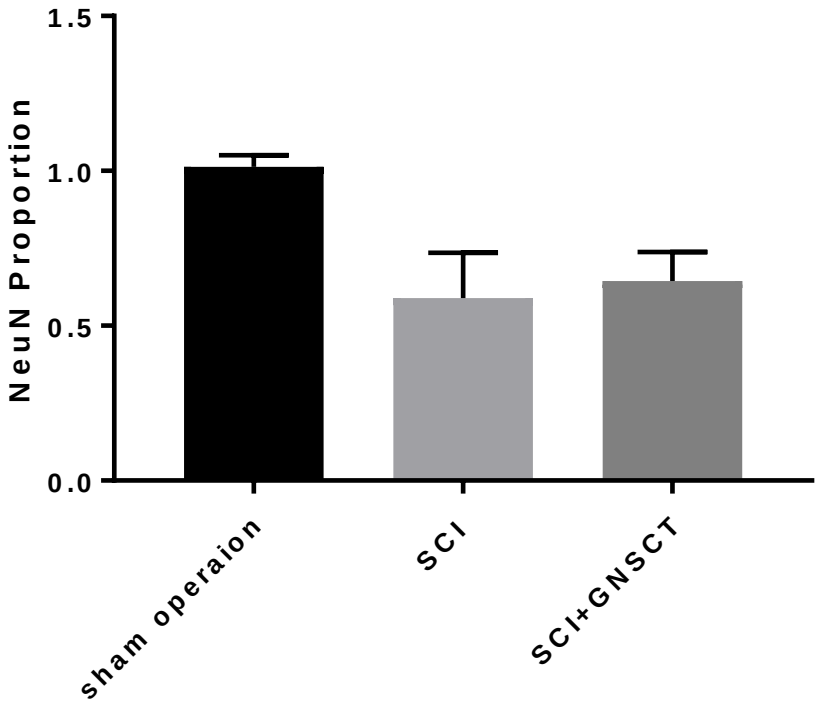


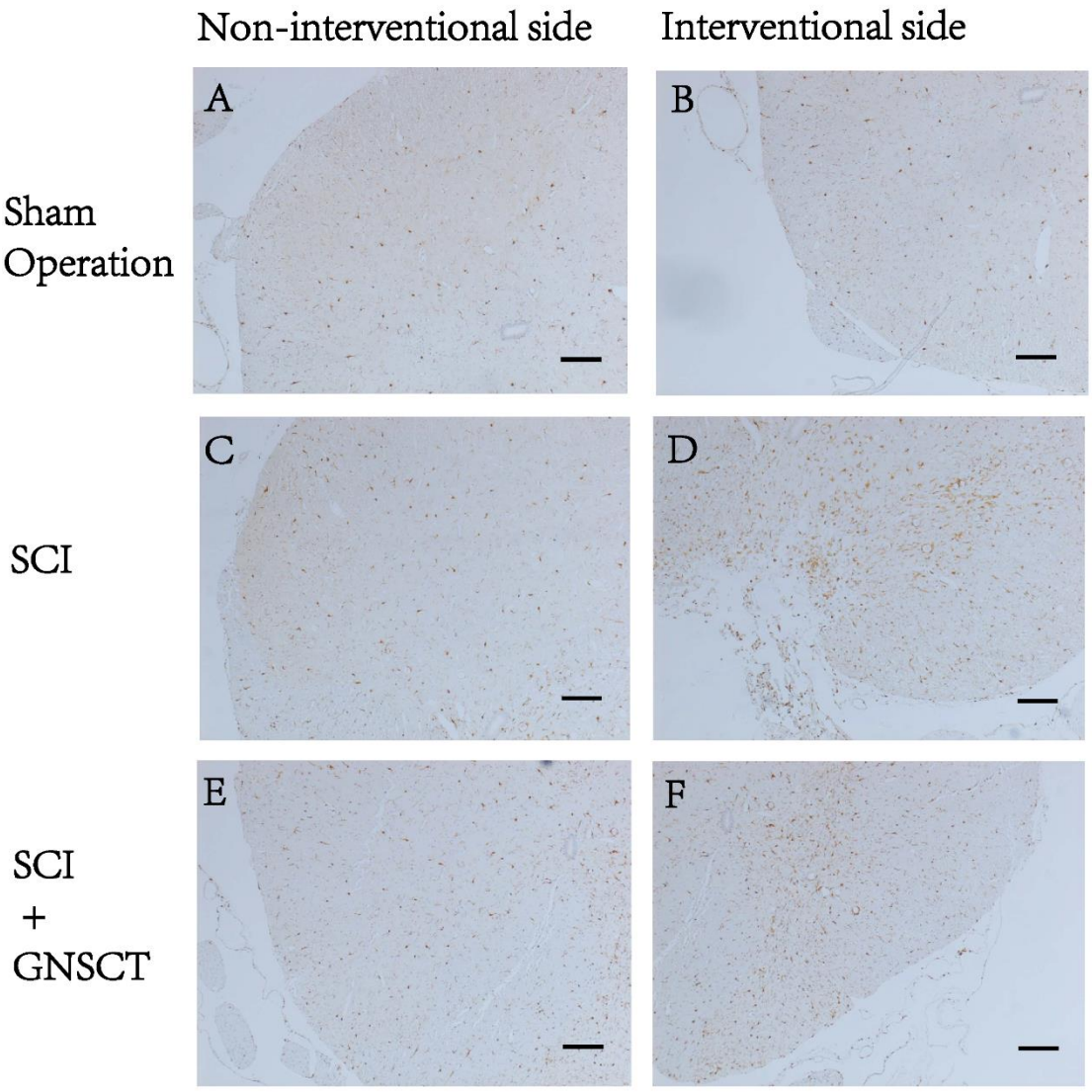
Figure 20. The column bar graph of NeuN proportion in the sham operation group, “SCI” group and “SCI + GNSCT” group.

NeuN Score = the number of NeuN- positive neurons in the area of interest

NeuN Proportion = NeuN Score of the interventional side / NeuN Score of the non-interventional side

As stated in the Figure 20, the NeuN Proportion of the group “sham operation” was remarkably higher than both the group of “SCI” ($P < 0.0001$) and “SCI + GNSCT” ($P < 0.0001$). Though, the numbers of neurons in “SCI + GNSCT” group was slightly larger than “SCI” group. There were no statistically significant differences in the NeuN Proportion between the group “SCI” and “SCI + GNSCT” ($P > 0.05$).

3.3.4 Iba-1 staining results



*SCI: spinal cord injury

#GNSCT: GABAergic neural stem cell transplantation

Figure 21. Photomicrograph shows Iba-1 positive microglial cells in the dorsal horn.

A. Photomicrograph of the non-interventional side of the group of sham operation;

B. Photomicrograph of the interventional side of the group of sham operation;

C. Photomicrograph of the non-interventional side of the group of "SCI";

D. Photomicrograph of the interventional side of the group of "SCI";

E. Photomicrograph of the non-interventional side of the group of "SCI + GNSCT";

F. Photomicrograph of the interventional side of the group of “SCI + GNSCT”

Scale bar, 200 μm.

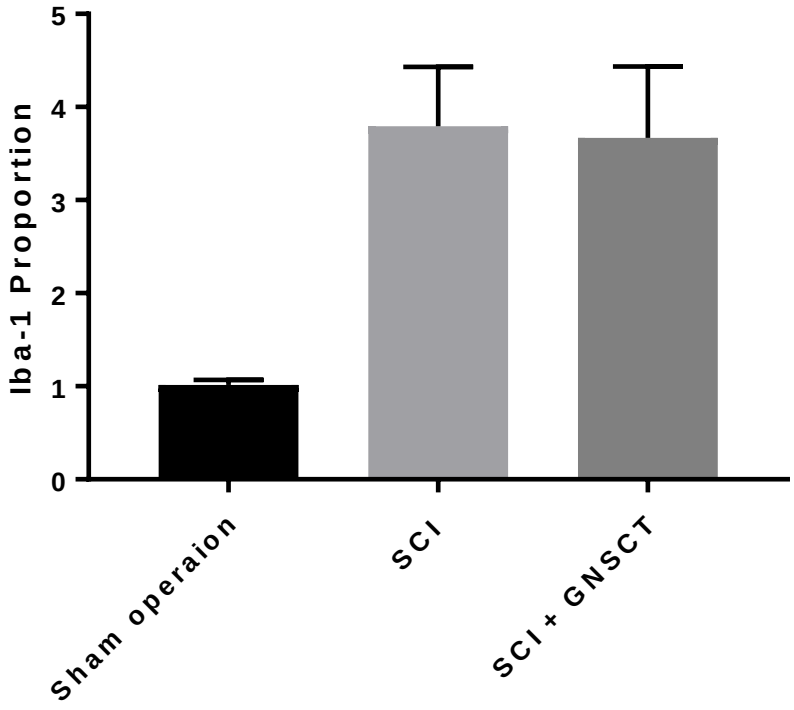


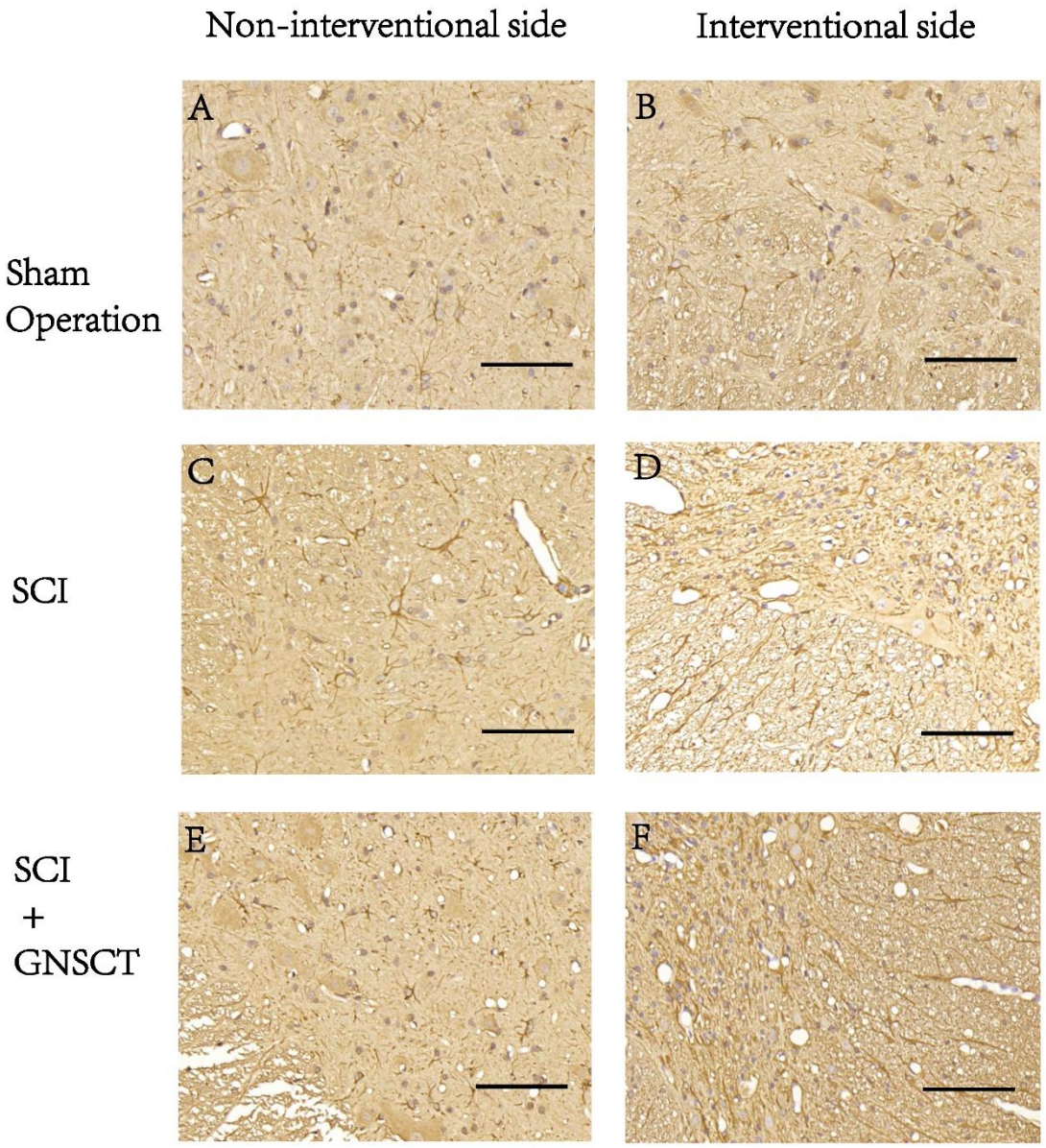
Figure 22. The column bar graph of Iba-1 proportion in the sham operation group, “SCI” group and “SCI + GNSCT” group.

Iba-1 Score = the amount of Iba-1 positive microglial cells in the area of interest

Iba-1 Proportion = Iba-1 Score of the interventional side / Iba-1 Score of the non-interventional side

As stated in the Figure 22, the Iba-1 Proportion of the group “sham operation” was significantly lower than both the group of “SCI” ($P < 0.0001$) and “SCI + GNSCT” ($P < 0.0001$). And no significant difference in the Iba-1 Proportion was detected between the group “SCI” and “SCI + GNSCT” ($P > 0.05$).

3.3.5 GFAP staining results



*SCI: spinal cord injury

#GNSCT: GABAergic neural stem cell transplantation

Figure 23. Photomicrograph shows GFAP positive astrocytes in spinal cord.

A. Photomicrograph of the non-interventional side of the group of sham operation;

B. Photomicrograph of the interventional side of the group of sham operation;

C. Photomicrograph of the non-interventional side of the group of "SCI";

D. Photomicrograph of the interventional side of the group of "SCI";

E. Photomicrograph of the non-interventional side of the group of "SCI + GNSCT";

F. Photomicrograph of the interventional side of the group of “SCI + GNSCT”

Scale bar, 100 μm.

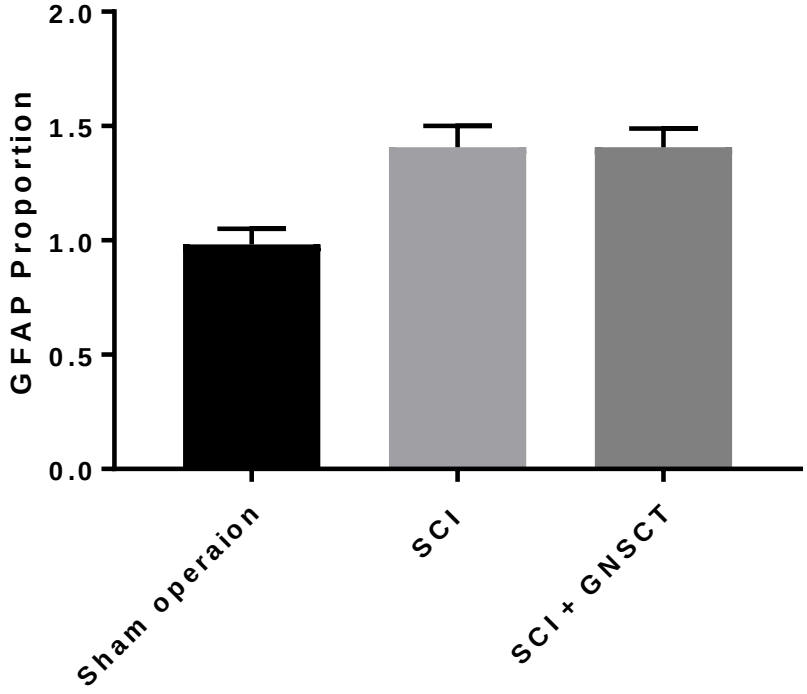


Figure 24. The column bar graph of GFAP proportion in the sham operation group, “SCI” group and “SCI + GNSCT” group.

GFAP Score = the amount of GFAP positive astrocytes in the area of interest

GFAP Proportion = GFAP Score of the interventional side / GFAP Score of the non-interventional side

As stated in the Figure 24, the GFAP Proportion of the group “sham operation” was significantly lower than both the group of “SCI” ($P < 0.0001$) and “SCI + GNSCT” ($P < 0.0001$). And no significant difference in the GFAP Proportion was detected between the group “SCI” and “SCI + GNSCT” ($P > 0.05$).

3.3.6 The results of correlation analysis between mechanical pain thresholds and IHC(Immunohistochemistry) intensity proportion of GABA

We investigated whether increases in GABA levels (in laminae I–II) following GABAergic neural stem cell transplantation accelerated the attenuation of pain-related behaviors. A significant positive correlation was observed between GABA levels and mechanical pain thresholds (Figure 25, $r = 0.9544$, $n = 45$, $p < 0.0001$). The result suggested that increased GABAergic tone (in laminae I–II) might lead to reducing neuropathic pain following SCI.

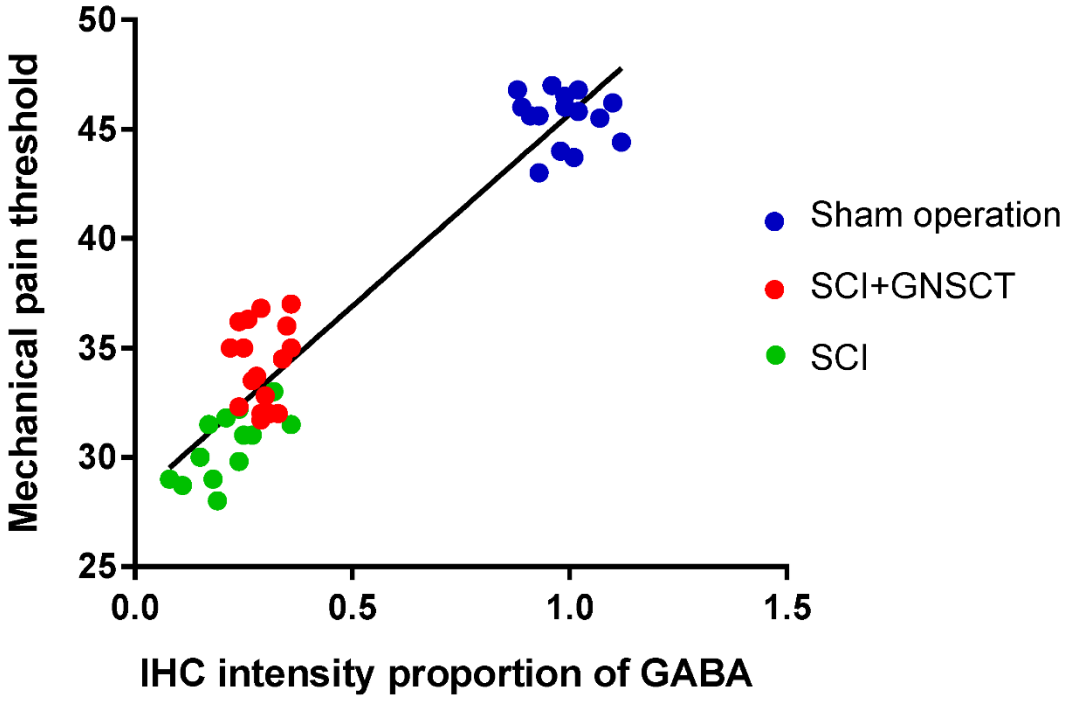


Figure 25. Relationships between the mechanical pain thresholds (assessed at 8 weeks after the second surgery) and the proportion of the immunohistochemistry intensity of GABA in the treated dorsal horns of rats in three groups. A significant positive correlation can be observed between the thresholds of the electric von Frey test and GABA intensity ($r = 0.9544$, $n = 45$, $p < 0.0001$). Blue, red, and green points indicate the values of rats in the sham operation group, "SCI" group and "SCI + GNSCT" group, respectively.

4. Discussion

4.1 The SCI animal models

The establishment of a stable and standardized animal model is necessary for the SCI investigation. Rodent species are the most widely used and probably best-suited animal models for studies of SCI (Sharif-Alhoseini et al., 2017). An ideal animal model is constructed under the guidance of mirroring all aspects of human SCI as well as generating a graded injury easily, consistently, and reproducibly. However, given the complexity of the SCI process in human beings, no model can meet these criteria perfectly (Cheriyan et al., 2014). In recent decades, animal models of contusion, distraction, compression, dislocation, transection, etc. are widely applied to study SCI. Contusion models are featured by using transient force to generate displacement and injury of the spinal cord. Electromagnetic, weight-drop, and air pressure devices are most commonly used in this kind of model. Distraction models are established by controlling the stretch of the cord to inflict graded injury. Compression models are characterized by the application of the spinal cord compression at a specific force and duration. Dislocation models are produced by utilizing lateral vertebral displacement to cause damage. Transection models mean that the spinal cord is transversely severed completely or partially at a particular level (Cheriyan et al., 2014). Generally, contusion and compression models approximate the biomechanics and pathology of human SCI in a better way, while transection models are easy to be established with satisfactory stability and reproducibility.

Therefore, the unilateral spinal cord hemisection rat model at T13 level was established to inflict injury and chronic neuropathic pain. This kind of dorsal horn hemisection animal model is characterized by the hyperexcitability of dorsal horn neurons (Kim et al., 2003). The model in this project generated stable and consistent neuropathic pain after surgery without motor dysfunction. According to the assessment of the BBB scale, we found that all rats after SCI achieved complete

functional recovery of locomotion in our experiment which results in minimal behavioral interference to the subsequent pain evaluation. Meanwhile, all rats after dorsal horn hemisection were observed with an obvious decrease in the mechanical pain threshold. This model seems to simulate a reliable myelopathic pain after SCI without causing other physical dysfunctions, and it lays a foundation for assessing the effectiveness of experimental therapeutic interventions. Moreover, given that hemisection contributes to a minimal injury to the rats' spinal cords, postoperative animal care is easier in this kind of models than in other models. In addition, this model produces a unilateral injury that provides the opportunity to compare the histological changes between the injured and healthy sides of the same rat.

4.2 Principles for immunohistochemical staining

Immunohistochemistry is a widely utilized method that combines anatomical, immunological and biochemical techniques for the application of immunostaining. The history of immunohistochemistry can be traced back to 90 years ago and the basis of this technique has been proposed since the 1930s. However, it was not until 1941 that Dr. Albert Coons first implemented FITC-labeled antibodies to detect Pneumococcal antigens in infected tissue sections which marked the birth of immunohistochemistry (Coons et al., 1941). Since then, this technique has witnessed many improvements over the decades and has become an essential tool in disease diagnosis and research applications (Teruya-Feldstein, 2010). The fundamental principle is using labeled antibodies selectively against target antigens in specific cells or tissues to demonstrate antigen-antibody binding with a colored and visible histochemical reaction (Magaki et al., 2019).

Usually, the technique comprises five phases: (1) The target tissue is collected, fixed and cut into sections to prepare complete samples maintaining cell morphology, tissue architecture and the antigenicity of target epitopes; (2) Proteolytic-Induced Epitope Retrieval (PIER) or Heat-Induced Epitope Retrieval (HIER) is performed to unmask

antigenic epitopes, making them more accessible to antibody binding; (3) The samples are incubated with blocking buffer to decrease nonspecific antibody binding, reducing background staining; (4) The samples are incubated with primary antibodies to form antigen-antibody complexes. Then signal detection was maximized via a reaction between labeled secondary antibodies and antigen-bound primary antibodies; (5) The target antigens are localized and visualized with chromogenic detection based on enzyme conjugation, while immunofluorescence detects the antigens using antibodies attached to fluorochromes.

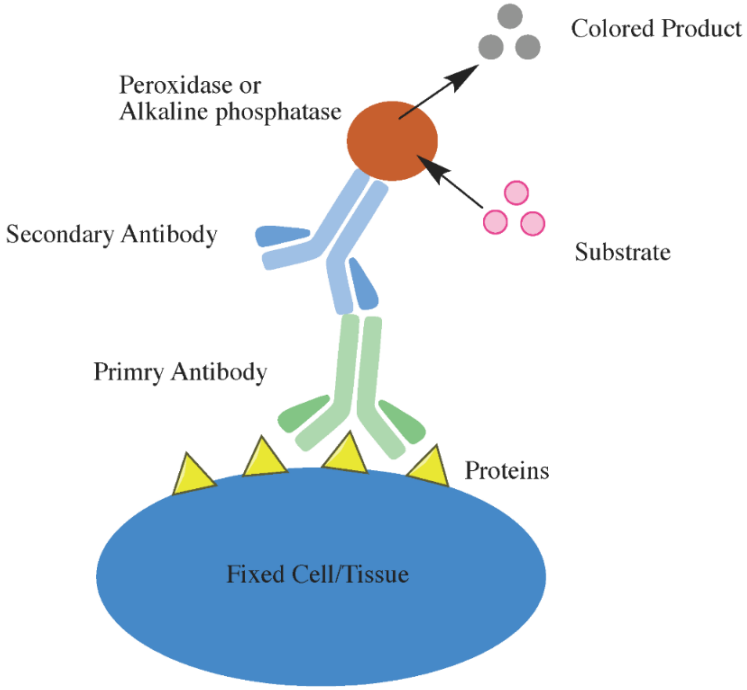


Figure 26. Scheme of the indirect immunohistochemistry

There are different approaches in immunohistochemistry methodology, but in the current work, DAB staining was used to detect target antigens in the spinal cord. The method of DAB staining has many advantages, such as simple operation, high signal sensitivity and low staining background. Besides, since DAB is insensitive to light, the staining results can be kept stable for many years. Moreover, the hematoxylin was applied as a second dye to provide a stain of whole cell that helps the DAB stain stand out (Cartun et al., 2013).

4.3 The spinal cord pathophysiological changes in rats receiving dorsal horn hemisection

Recently, multiple rodent model-based studies were executed to study the pathophysiological changes of SCI (Nardone et al., 2017). Commonly, the spinal cord injury responses can be divided into acute, secondary, and chronic injury phases. Specifically, spinal cord injury immediately causes hemorrhage and cell death and the following multiple cascades result in delayed damage to tissue. This indicates the begin of the secondary phase. The injury to the nervous tissue site initiates a cascade of neuroinflammatory reactions resulting in the concentration and activation of immune cells, as well as the release of numerous inflammatory cytokines. The main features of the secondary phase contain edema, apoptosis, ionic imbalance, neurotransmitters accumulation, free-radical production, lipid peroxidation, inflammation, demyelination, and glial scar launch. As time goes, the chronic injury phase begins with apoptosis, demyelination, cavity formation, glial scar maturation, and regenerative processes (Couillard-Despres et al., 2017, Yuan and He, 2013).

In our work, the rats in the "SCI" group and the "SCI + GNSCT" group were sacrificed at the 9th week after hemisection injury, and the spinal cord tissue was removed for further IHC staining. The NeuN is a common biomarker for neurons and the results of NeuN immunohistochemistry staining indicated a reduction in the number of neurons after SCI. Additionally, the GFAP is specifically and abundantly expressed in astrocytes of the CNS, which is considered to involve in the structure of the cytoskeleton and thus maintain the morphology of astrocyte (Tykhomyrov et al., 2016). The results of GFAP immunostaining suggested the upregulation of GFAP and the proliferation of astrocytes in the chronic process of SCI. Besides, the Iba-1 is highly and specifically expressed in macrophages/microglia (Imai et al., 1996). The IHC results in the injured side demonstrated the overexpression of Iba-1 as well as the increase in the number of microglia when compared with the contralateral side. The enhanced GFAP and Iba-1 immunoreactivity in the region of injury of dorsal horn

revealed the glial activation in the process of SCI. Furthermore, there was no statistically important difference in the level of glial activation between the “SCI” group and the “SCI + GNSCT” group, which indicated that the neural stem cell transplantation did not suppress the inflammatory overreaction in hemisection rat models of our project.

Besides, the results of NeuN, GFAP and Iba-1 staining suggested a chronic phase of SCI with the features of neuron loss, astrocytosis and microglia hyperplasia in the area of the lesion. The pathological changes in the chronic phase of our project were similar to those of the SCI models in other studies (Kjell and Olson, 2016, Hulsebosch, 2002). However, in this experiment, the depth of hemisection injury was controlled at about 1mm, causing relatively small damage to the spinal cord. Therefore, the injury site was limited to the posterior horn without any injury to the anterior horn and there was no formation of cyst or cavity.

4.4 Neural stem cells transplantation for the treatment of SCI

SCI usually leads to persistent motor dysfunction and chronic neuropathic pain. At present, the commonly used clinical pharmacotherapy displays limited effectiveness for the relief of neuropathic pain, and the systemic medication adverse side effects are inevitable (Alles and Smith, 2018). It is believed that the CNS neurons have a poor capacity of intrinsic regeneration, which limits the tissue repair at the injury site and the restoration of neural function. Therefore, a variety of researches has been performed to find methods to improve neuron regeneration (Cartoni et al., 2019). Currently, neural stem cell transplantation is regarded as a promising therapeutic approach for the treatment of SCI. According to recent studies, the most commonly proposed mechanisms by which transplanted cells promote repair and functional improvements may include the following five points: (1) Neuroprotection: cellular transplant can mitigate the secondary injury of SCI to protect endogenous cells; (2) Immunomodulation: cell transplantation can attenuate detrimental inflammation or

promote conducive inflammatory reaction to provide benefits; (3) Axon regeneration: cell transplantation may promote bridge/relay formation and modify astrocyte response to facilitate the repair of neural connections ; (4) Neuronal relay formation: cell transplantation might promote the formation of relay circuits between transplanted neurons and descending axons; (5) Myelin regeneration: the transplanted neural stem cells, Schwann cells, or oligodendrocyte precursor cells (OPCs) can produce oligodendrocytes, accordingly promoting the regeneration of myelin sheaths (Assinck et al., 2017). In the case of neuropathic pain, cell transplantation can replace the dead or injured neurons, stimulate axon growth and neuronal connectivity, as well as promote re-myelination, which can contribute to the alleviation of neuropathic pain following SCI.

4.4.1 The cell types for transplantation

Multiple kinds of cells have been assessed as candidate cell types for the transplantation to treat SCI, such as neural stem and progenitor cells (NSPCs), olfactory ensheathing cells (OECs), mesenchymal stem cells (MSCs) and OPCs. It has been demonstrated in previous studies that NSPCs are pluripotent and self-renewing cells, which can differentiate into all types of neural cells (Walker et al., 2016, Goldman, 2005). OECs are a type of glial cells that provide the neurotrophic substrate to support axon growth and facilitate remyelination (Ruitenberg et al., 2006). MSCs are multipotent stromal cells with the capability of cell differentiating into cells such as osteoblast, myocyte, adipocyte and neuron.

However, as stated before, many studies show that the changes in GABA expression after SCI would lead to the dysfunction of the inhibitory system through multiple mechanisms, therefore promote the development of pain behavior. Given the results of these studies, a hypothesis was proposed that transplantation of GABAergic neural stem cells may increase post-synaptic inhibitory potentials, consequently reverse the neural hyperexcitability in the dorsal horn following SCI. Hence, in this project, the

GABAergic precursor cells were isolated from E14 embryo rats and applied to the cell transplantation treatment for relieving neuropathic pain after SCI.

4.4.2 The timing of cell transplantation

The timing for cells transplantation has significant effects on functional outcomes for SCI treatment. Li and colleagues found that graft at the subacute stage after spinal cord transection contributes to better efficiency of the treatment when compared with the acute phase or chronic phase (Li et al., 2011). Likewise, other reports also revealed that cell transplantation in the subacute phase appears to gain the best functional recovery of SCI (Cheng et al., 2017, Mothe and Tator, 2013). The poor effect of transplantation in the chronic phase may be due to the formation of glial scar at the site of the lesion, and the impaired cellular function in this period leads to the partial irreversible nature of injuries (Oyinbo, 2011). Moreover, since the acute phase inflammatory reactions of SCI produce a cytotoxic environment, which is not conducive to the survival and growth of the transplanted cells, the transplantation effect in the acute phase is slightly worse than that in the subacute phase (Mothe and Tator, 2013). In this study, we transplanted GABAergic stem cells into hemisected rats' spinal cords on the 7th day after the injury, which might be an optimal time of cell transplantation for SCI treatment.

4.4.3 GABAergic neural stem cell transplantation in attenuating SCI-induced neuropathic pain

In the CNS, the gamma-aminobutyric acid acts as an important inhibitory neurotransmitter and GABA neurons in the dorsal horn are major targets for the pain regulation.

In our project, we transplanted the GABAergic neural stem cells into the lesion of the

dorsal horn to recover the function of the GABA inhibitory system after SCI. The results of GABA and GAD immunostaining suggested increased levels of GABA and GAD in the damaged side of the dorsal horn in the "SCI + GNSCT" group. Besides, according to the results of von Frey pain assessment, the GABAergic neural stem cell transplantation promotes an efficient relief of SCI-induced neuropathic pain. What's more, the pain-relieving effect of cell transplantation began in the second week and reached a peak at 6th week after the transplantation (about 70% of the full recovery level), and the effect maintained until the end of this experiment. Many other studies on GABAergic neural stem cell transplantation therapies for SCI have also obtained similar findings. For example, Fandel's team transplanted human stem cell-derived interneuron precursors into the injured mice's spinal cord, which mitigated mice's pain-related behaviors induced by SCI (Fandel et al., 2016). Furthermore, Bráz and colleagues had found that transplantation of telencephalic GABAergic precursors overcome the dorsal hyperexcitability, reducing the injury-induced neuropathic pain (Bráz et al., 2012).

The popular hypothesis regarding the mechanism of neuropathic pain mitigation is that the enhanced level of GABA expression after GABAergic neural stem cell transplantation would ease the pain. The transplanted GABAergic neuronal precursors can differentiate into GABAergic neuron subtype cells and establish a synaptic connection with the original neurons to form a neural network, thereby rebuilding the inhibitory function of GABA neurons (Etlin et al., 2016, Bráz et al., 2012). There is another theory that the implanted neural stem cells generate a neuroprotective function, which can reduce inflammatory cytokines, reactive oxygen species and cell death (Yousefifard et al., 2016). Nevertheless, the suppression of the inflammatory responses was not observed after transplantation in this project. There was no important difference in the level of glial activation between the "SCI" group and the "SCI + GNSCT" group. Since the limited sample size of our work, further studies employing a larger cohort are still needed.

4.5 Research limitations

In this project, we only monitored the mechanical pain threshold. It is better to record the thermo-pain threshold in further investigations to improve pain assessment.

What's more, the transplanted cells were not labeled with cell tracers prior to the transplantation, so it was more difficult to identify the migration, survival, and differentiation of the transplanted stem cells in the spinal cord.

In addition, manual transection surgery is not able to assure that the spinal cord injuries generated in different batches share the totally same features. This problem however is common to all animal injury models of the spinal cord.

5. Summary

5.1 Summary

Background and objective: Spinal cord injury (SCI) generally results in neurological deficits involving permanent motor dysfunction and refractory neuropathic pain. Recent researches have indicated that the impaired function of the Gamma-aminobutyric acid (GABA) inhibitory system is one of the key mechanisms leading to enhanced pain following SCI. We examined the impact of GABAergic neural stem cell transplantation (GNSCT) on the possible reduction of neuropathic pain in a spinal cord hemisection rat model.

Methods: In this project, forty-five Sprague Dawley rats were randomly divided into three groups (i.e. Group "sham operation", "SCI" and "SCI + GNSCT"). Rats in the "sham operation" group underwent only a laminectomy, while the other rats in the "SCI" and "SCI + GNSCT" groups underwent an additional lateral dorsal horn hemisection. One week after the injury, the rats in the "sham operation" group underwent only an opening of the spinal wound, while those in the groups "SCI" and "SCI + GNSCT" underwent transplantation of either Phosphate-buffered saline (PBS) or GABAergic neural stem cells into the site of the previous lesion. Eight weeks later, all rats were sacrificed and their spinal cords were extracted for further staining. The Basso, Beattie and Bresnahan (BBB) locomotor rating scale was used to assess the motor function of the rats. An electronic von Frey apparatus was applied to evaluate the pain hypersensitivity of the animals before and after the surgery. The immunohistochemical staining images were analyzed to evaluate the morphological changes of each spinal cord after transplantation.

Results: The BBB Scores of the hindlimbs eventually recovered to normal levels in all three groups. Eight weeks after the stem cell transplantation, the pain threshold of the sham operation group maintained at 45.5, the pain threshold of the PBS transplantation group reached 30, and the pain threshold of the cell transplantation

group recovered to 34.5. The results of immunohistochemical staining indicated higher levels of GABA ($P < 0.05$) and Glutamic acid decarboxylase (GAD) ($P < 0.05$) in the "SCI + GNSCT" group than in the "SCI" group. A significant positive correlation could be observed between the thresholds of the electric von Frey test and the GABA intensity ($r = 0.9544$, $n = 45$, $p < 0.0001$). Nevertheless, there were no significant differences in the proportion of NeuN between the group "SCI" and "SCI + GNSCT" ($P > 0.05$). Besides, no statistically significant difference in the proportion of Ionized calcium-binding adapter molecule 1 (Iba-1) ($P > 0.05$) and Glial Fibrillary Acidic Protein (GFAP) ($P > 0.05$) was detected between the group "SCI" and "SCI + GNSCT".

Conclusion: In this study, the GABAergic stem cell transplantation could attenuate the SCI-induced neuropathic pain via partially recovering the GABA inhibitory function. These results might have been even more obvious if the transplanted cells would have been labeled pre-operatively.

5.2 Zusammenfassung

Hintergrund und Zielsetzung: Eine Rückenmarksverletzung (SCI) führt häufig zu schweren neurologischen Ausfällen, wie Lähmungen, vegetativen Störungen und sehr oft auch zu ausgeprägten chronischen neuropathischen Schmerzen. Jüngste Studien haben gezeigt, dass eine Beeinträchtigung des Gamma-Aminobuttersäure (GABA) -Hemmungssystems einer der Hauptmechanismen für die Zunahme von Schmerzen nach SCI ist. Wir haben in einem Rückenmark-Hemisektions-Rattenmodell untersucht, inwieweit eine GABAerge neurale Stammzelltransplantation (GNSCT) eine mögliche Reduktion chronischer neuropathischer Schmerzen bewirken kann.

Methoden: In dieser Studie wurden 45 Sprague Dawley-Ratten in drei Gruppen eingeteilt (d. H. Gruppe "Sham", "SCI" und "SCI + GNSCT"). Ratten der "Sham" Gruppe erhielten lediglich eine Laminektomie, während bei den anderen Ratten der Gruppe "SCI" und "SCI + GNSCT" zusätzlich eine laterale dorsale Hemisektion des Rückenmarkes durchgeführt wurde. Eine Woche nach der ersten Operation wurde bei den Ratten der "Sham" Gruppe lediglich die spinale Wunde wieder eröffnet, während bei den Gruppen "SCI" und "SCI + GNSCT" entweder PBS- oder GABAerge neurale Stammzellen im Bereich der vormaligen Läsion transplantiert wurden. Acht Wochen später wurden alle Ratten geopfert und ihr Rückenmark zur weiteren Färbung extrahiert. Zur Beurteilung der Motorik der Ratten benutzten wir die Skala von Basso, Beattie und Bresnahan (BBB). Die Schmerzüberempfindlichkeit vor und nach der Operation untersuchten wir bei den Ratten mit einem elektronischen von Frey-Apparat. Zur Untersuchung der histopathologischen Veränderungen des Rückenmarks der Ratten verwendeten wir immunhistochemische (IHC) Verfahren.

Ergebnisse: Die BBB-Werte für die Hinterbeine der Ratten erholten sich bei allen drei Gruppen und erreichten wieder Normalwerte. Acht Wochen nach Stammzelltransplantation hatte die Schmerzschwelle bei der Shamoperationsgruppe wieder einen Wert von 45,5 erreicht, bei der PBS-Transplantationsgruppe betrug der Wert 30 und bei der der Zelltransplantationsgruppe war der Wert auf 34,5 angestiegen. Die Ergebnisse der IHC zeigten höhere GABA- ($P < 0,05$) und GAD-Werte ($P < 0,05$)

in der Gruppe "SCI + GNSCT" als in der Gruppe "SCI". Eine signifikante positive Korrelation konnte zwischen den Schwellenwerten des elektrischen von Frey-Tests und der GABA-Intensität beobachtet werden ($r = 0,9544$, $n = 45$, $p < 0,0001$). Es gab jedoch keinen signifikanten Unterschied bei dem NeuN-Anteil zwischen der Gruppe "SCI" und "SCI + GNSCT" ($P > 0,05$). Außerdem wurde kein signifikanter Unterschied beim Iba-1-Anteil ($P > 0,05$) und dem GFAP-Anteil ($P > 0,05$) zwischen der Gruppe "SCI" und "SCI + GNSCT" festgestellt.

Schlussfolgerung: In dieser Studie konnte gezeigt werden, dass die GABAerge Stammzelltransplantation den SCI-induzierten neuropathischen Schmerz durch teilweise Wiederherstellung der GABA-Hemmfunktion abschwächen kann. Die Ergebnisse hätten jedoch durch eine entsprechende präoperative Markierung der transplantierten Zellen noch deutlicher nachgewiesen werden können.

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9. Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde am Hertie Institut und Klinik für Neurochirurgie unter Betreuung von Prof. Dr. med. MH Morgalla durchgeführt.

Die Konzeption der Studie erfolgte durch Prof. Dr. med. MH Morgalla .

Sämtliche Versuche wurden nach Einarbeitung durch Labormitglieder und Prof. Dr. med. MH Morgalla von mir mit Unterstützung durch Prof. Dr. med. MH Morgalla durchgeführt.

Die statistische Auswertung erfolgte nach Anleitung durch Prof. Dr. med. MH Morgalla druchgeführt.

Ich versichere, das Manuskript selbständig (nach Anleitung durch Prof. Dr. med. MH Morgalla) verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

Unterschrift

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Sprache

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