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GABAergic neural stem cells transplantation after spinal cord injury induced chronic neuropathic pain in a rat model

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To my parents

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Abbreviations

SCI	Spinal Cord Injury
CNS	Central Nervous System
CNPSCI	Chronic Neuropathic Pain secondary to Spinal Cord Injury
IASP	International Association for the Study of Pain
ISCIPBDS	International Spinal Cord Injury Pain Basic Data Set
NLI	Neurological Level of Injury
DRG	Dorsal Root Ganglion
PAG	Periaqueductal Gray
RVM	Rostral Ventral Medulla
SP	Substance P
CGRP	Calcitonin Gene-related Peptide
SMC	Smooth Muscle Cell
ALT	Anterolateral Tract
GABA	Gamma Aminobutyric Acid
GAD	Glutamate Decarboxylase
NPY	Neuropeptide Y
PAF	Primary Afferent Neuron
NGF	Nerve Growth Factor
BDNF	Brain Derived Neurotrophic Factor
VGSC	Voltage Gated Sodium Channel
VGCC	Voltage Gated Calcium Channel
NMDA receptor	N-methyl-D-aspartate receptor
CCK	Cholecystokinin
TNFα	Tumor Necrosis Factor Alpha

OECs	Olfactory Enscheathing Cells
MSCs	Mesenchymal Stem Cells
FSCs	Fetal Stem Cells
iPSCs	Induced Pluripotent Stem Cells
PID	Post Injury Day
MGE	Media Ganglionic Eminence
LGE	Lateral Ganglionic Eminence
POA	Preoptic Area
RA	Retinoic Acid

1. Introduction

As a worldwide disease, spinal cord injury (SCI) is associated with an annual morbidity of 15-40 cases per million population, including approximately 45% of complete SCIs (Sekhon and Fehlings 2001). According to the latest report by the US National Spinal Cord Injury Statistical Center, SCI frequently occurs at the age group of 16-30 years (mean 33.3 years); typically, 81% cases are found in men(French, Campbell et al. 2007). SCI is a devastating neurological condition, which generally results in marked loss of motor function and feeling below the injury level, as well as chronic pain in most patients. About 70% SCI patients have suffered from chronic pain (Stormer, Gerner et al. 1997, Siddall and Loeser 2001), which can lead to a severe cognitive problems and decrease in the quality of life of the patients. Moreover, SCI would also cause great burdens on both the families and the society (Westgren and Levi 1998, Andresen, Biering-Sorensen et al. 2016). Unfortunately, no totally successful treatment is available at present, and there are still many limitations regarding drug therapies, interventional measures or other treatments (such as acupuncture) (Chong and Bajwa 2003, O'Connor and Dworkin 2009, Magrinelli, Zanette et al. 2013).

Therefore, the current study aimed to explore the effect of post-SCI GABAergic stem cell transplantation on relieving the chronic neuropathic pain after SCI.

1.1. Neuropathic Pain after SCI

According to the International Association for the Study of Pain (IASP), neuropathic pain is defined as a somatosensory nervous system disorder (Treede, Jensen et al. 2008), which can be caused by neural damage or disease. Neuropathic pain, which is generally chronic and refractory, is associated with the morbidity of 7%-8% of the population (Jensen, Gottrup et al. 2001, Torrance, Smith et al. 2006, Bouhassira, Lanteri-Minet et al. 2008, Gilron, Baron et al. 2015).

Neuropathic pain displays numerous clinical symptoms, including trigeminal neuralgia and herpetic zoster neuralgia. Moreover, neuropathic pain can be induced by a variety of

factors; for instance, metabolic problems, vascular disease, injury and infection can give rise to peripheral neuropathic pain. In contrast, spinal cord injury (SCI), cancer and multiple sclerosis are the leading causes of central neuropathic pain (Jones, Lawson et al. 2016). Typically, around 40% of the patients would suffer from neuropathic pain after chronic SCI (Werhagen, Budh et al. 2004), which will reduce the quality of life of SCI patients (Jensen, Chodroff et al. 2007). Specifically, the neuropathic pain at the levelof injury shows up probably earlier than the pain below the level of injury and it cannot be alleviated with time (Siddall, McClelland et al. 2003). The clinical manifestations of chronic neuropathic pain secondary to spinal cord injury (CNPSCI) are hyperalgesia and allodynia, which often manifest as burning, shooting or pricking sensations(Finnerup, Johannesen et al. 2001). Meanwhile, there may be fracture, spinal nerve compression or injury of the soft tissue after SCI. Thus, the pain may also be different (Cardenas and Felix 2009). According to the International Spinal Cord Injury Pain Basic Data Set (ISCIPBDS), pain after SCI can be classified into musculoskeletal (nociceptive), viscereal (nociceptive), other (nociceptive), at-level SCI (neuropathic), below-level SCI (neuropathic), other (neuropathic), other and unknown (Widerstrom-Noga, Biering-Sorensen et al. 2008, Cardenas and Felix 2009, Widerstrom-Noga, Biering-Sorensen et al. 2014).

Table 1. Classification of pain after SCI (Widerstrom-Noga, Biering-Sorensen et al. 2014)

Musculoskeletal (nociceptive)	Pain originating in the musculoskeletal structures.
Visceral (nociceptive)	Pain locating in the thorax, abdomen or pelvis and originating in visceral structures.
Other (nociceptive)	Nociceptive pain that is not classified into musculoskeletal or visceral pains.
At-level SCI (neuropathic)	Pain located within or at three dermatomes below the neurological level of injury (NLI)
Below-level SCI (neuropathic)	Pain locating at three dermatomes below the NLI.
Other (neuropathic)	Neuropathic pain locating above, at, or below the NLI but not directly related to SCI.
Other	Pain unrelated to SCI and no induced by any identifiable noxious stimulus, inflammation or damage to the nervous system.
Unknown	Pain of unknown etiology.

1.2. Anatomical physiology of neuropathic pain

1.2.1. Nociceptors

Nociceptors are the subpopulations of peripheral afferent nerve fibers, which can detect the thermal , as well as mechanical and chemical stimuli reaching the noxious range. Specifically, the dorsal root ganglia (DRG) connected to the spinal cord are the cell bodies of nociceptors, which can conduct the pain signals from the whole body except for the face. Besides, the trigeminal ganglia are also the cell bodies of nociceptors, which can conduct the pain signals from the face. There are two types of nociceptors, including the myelinated fibers and the unmyelinated fibers (Light and Perl 1979). Of them, the myelinated fibers include Aδ (responding to touch or hair movement) and Aβ

fibers (low-threshold mechanoreceptors). These fibers, which have large diameters, can conduct signals at a very fast speed and are mainly associated with the fast pain or “first pain”. On the other hand, the unmyelinated fibers include C fibers with a small diameter (0.2-1.5μm) and slow signal conduction speed. Therefore, they are related to the slow pain or “second pain” (Basbaum, Bautista et al. 2009, Todd 2010, Lewin and Nykjaer 2014, Benarroch 2016). Meanwhile, C fibers can also be divided into two groups, namely, non-peptidergic fibers (related to the skin) and peptidergic fibers (related to other tissues and the deeper regions of the skin) (Plenderleith and Snow 1993, Taylor, Peleshok et al. 2009).

The noxious pain signal generated by nociceptors will pass through the laminae I and laminae V, pass through the thalamus or amygdala, and reach the cerebral cortex. The somatosensory cortex can then detect the location and intensity of pain, and the pain signal will later cause emotional changes after it is conducted to the cingulate cortex and insular cortex (Yalcin, Barthas et al. 2014). Afterwards, the pain sensation can be generated under the cooperation of the cerebral cortex, thalamus and limbic system. Notably, both periaqueductal gray (PAG) and rostral ventral medulla (RVM) play vital roles in the descending pathways (Chuquilin, Alghalith et al. 2016).

At the same time, there is also another classification of nociceptors into thermal, mechanical and chemical nociceptors depending on the types of stimulus. Amongst them, thermal nociceptors can be activated by a heat or cold stimulus. The thermo-transient receptor potential channels (Thermo-TRP channels), which have over 6 subtypes, are related to this pain process. Typically, temperature higher than 43°C can activate TRPV1, which can also detect the chemical noxious stimulus (Khomula, Viatchenko-Karpinski et al. 2013, Labuz, Spahn et al. 2016, Nozadze, Tsiklauri et al. 2016); whereas TRPM8 is related to the cold sensation (Dhaka, Earley et al. 2008, Liu and Jordt 2018). Besides, mechanical nociceptors respond to mechanical stimuli, among which, TRPA1 is involved in this mechanical nociception mechanism (Fischer, Tambeli et al. 2008, Nozadze, Tsiklauri et al. 2016).

Furthermore, nociceptors also take part in the inflammatory and immune processes to regulate the pain, which is achieved through releasing neuropeptides and neurotransmitters from the peripheral terminals. Concretely, both calcitonin gene-related peptide (CGRP) and Substance P (SP) are released in the peripheral nerve terminals in the presence of calcium influx, which is induced by the noxious signal. Of them, CGRP contributes to muscle relaxation and vasodilation through activation of the RAMP1/CalcRL receptor in vascular smooth muscle cells (VSMCs). In addition, it can also enhance IL17 production by Th17 Cells and γδT cells in the psoriasis-like inflammation. In contrast, SP can activate tachykinin receptor 1 and 2 (TACR1/2) in vascular endothelial cells (VECs), which can thereby increase the vascular permeability, resulting in edema formation.(Pinho-Ribeiro, Verri et al. 2017)

1.2.2. Functions of interneurons in the dorsal horn circuits

There are two main types of dorsal horn neurons, including projection cells that have axons to project signals to the brain, and interneurons whose axons remain in the spinal cord. The pain-related projection neurons mostly belong to the anterolateral tract (ALT), and most ALT projection cells are in lamina I, while few of them are in lamina III – VI. They can project pain signals to the thalamus (through the spino-thalamic tract), reticular formation (through spino-reticular fibers) and to the periaqueductal grey matter of the midbrain. Moreover, interneurons can be mainly categorized into two types, namely, the inhibitory interneurons and excitatory interneurons. Of them, the inhibitory interneurons can utilize the GABA or glycine as a transmitter (Todd and Sullivan 1990). The GABAergic interneurons are shown to distribute in the laminae I-III through immunocytochemistry using GABA antibodies and the GABA synthesized enzyme glutamate decarboxylase (GAD) (Mitchell, Gentet et al. 2007, Polgar, Durrieux et al. 2013). Alternatively, the distribution of inhibitory interneurons in the spinal cord can also be defined using Pax2 and Lmx1b (Kardon, Polgar et al. 2014, Foster, Wildner et al. 2015, Gutierrez-Mecinas, Bell et al. 2017). Importantly, inhibitory interneurons, which

can release various neuropeptides (such as for nociception: neuropeptide Y (NPY), opioid peptides: enkephalin, dynorphin and galanin(Rowan, Todd et al. 1993, Duan, Cheng et al. 2014)), are shown to play a critical role in maintaining the balance between excitation and inhibition.

The dorsal horn gray matter in the spinal cord is separated into ten layers. Among them, Lamina I-X are surrounded by white matter (Rexed 1952), but only Lamina I-VI are related to neuropathic pain. Moreover, Lamina I-VI consist of the posteriordorsal horn, while Lamina I-III can collect most of the nociceptive afferent signals (Fig.1)(Todd 2010). Lamina I, where the Aδ nociceptor signal passes through (Dhaka, Earley et al. 2008), is the margin of the gray matter consisting of the marginal nucleus of spinal cord. Most neurons in this layer are related to the noxious and innocuous stimuli, and some neurons can specifically receive the noxious stimuli. Lamina II, also called substantia gelatinosa of Rolando, is the layer below Lamina I, which contains small interneurons and unmyelinated fibers to conduct the slow pain signal. To be specific, mechanical C fibers mainly conduct signals into this lamina(Liu, Vrontou et al. 2007, Seal, Wang et al. 2009). Lamina I and Lamina II are both the outer layer that is called superficial dorsal horn. There are more myelinated fibers in Lamina III, but the interneurons in this layer can only conduct the innocuous mechanical stimuli. Lamina IV is similar to Lamina III, but is thicker and can receive the innocuous stimuli together with Lamina III. These two layers are comprised of the nucleus proprius. Notably, Lamina V is the widest layer, where interneurons can conduct the pain signal to the brain stem, thalamus and cerebellum. Besides, Lamina III-V can collect both the noxious and innocuous stimulus signal from the Aβ fibers. Finally, Lamina VI is the base of the dorsal horn, which contributes to the fast pain flexion reflex(Todd 2010, von Hehn, Baron et al. 2012, Duan, Cheng et al. 2014, Benarroch 2016).

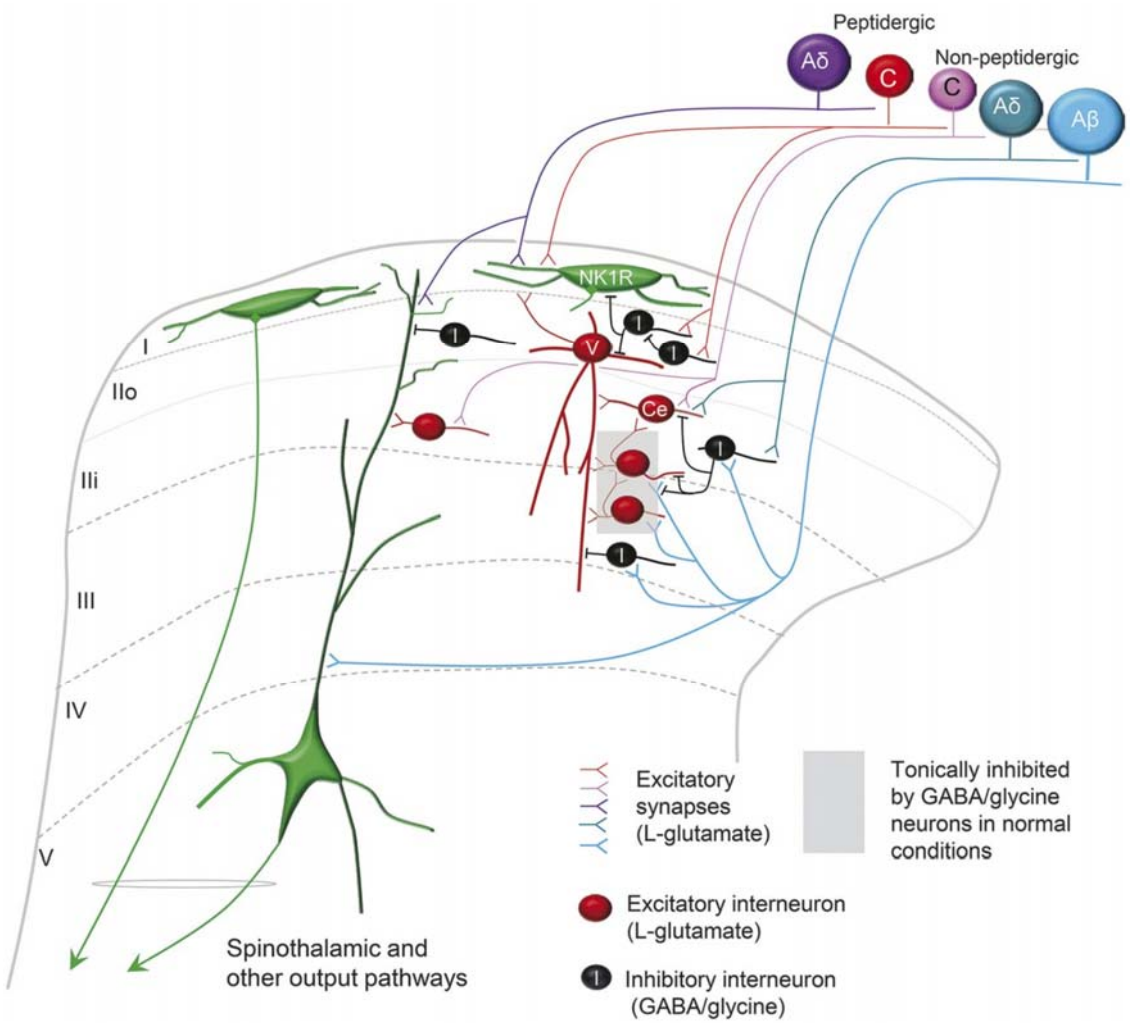

Fig.1. Dorsal horn and primary afferent inputs (Benarroch 2016).

1.2.3. Peripheral mechanisms of neuropathic pain

The peripheral mechanism is mainly about the decreased pain threshold and increased excitability of nociceptors. On this basis, innocuous stimuli can cause a painful sensation called allodynia. Alternatively, a normally slight pain feeling aggravates to an extremely pain that is referred to as hyperalgesia.(Treede, Meyer et al. 1992). Both of them may take place after injury of the primary afferent neurons (PAF). Typically, there exist many different theories about this peripheral sensitization. (Millan 1999, Ito, Okuda-Ashitaka et al. 2001, Sommer and Kress 2004).

Damage of the nerve fibers will lead to a local inflammatory reaction. As a result, the

“inflammation soup” can be released from the nociceptors or other cell types, which is comprised of histamine, bradykinin, neurotrophins, adenosine triphosphate, serotonin, nerve growth factor (NGF), cytokines (IL-1 ß, TNF-α, IL-6), calcitonin-gene related peptide (CGRP), substance P (SP) and brain-derived neurotrophic factor (BDNF). These factors can infiltrate into the injured area, which facilitate the vasodilatation and regulate the inflammatory response in mast cells, basophils, macrophages, neutrophils, endothelial cells and fibroblasts. Moreover, they may also increase the sensitivity of neurons to nociceptor stimuli (Willis and Westlund 1997, Millan 1999, Ito, Okuda-Ashitaka et al. 2001, Sommer and Kress 2004).

Besides, the sympathetic nervous system may also be involved in the formation of neuropathic pain. After PAF injury, the sympathetic axons in PAF and DRG areas can form the baskets around the sensory neurons, which will increase the ectopic activity of the DRG cells (Devor, Wall et al. 1992, Amir and Devor 1993, McLachlan, Janig et al. 1993).

Concretely, changes in the ion channels may be mainly responsible for the neuropathic pain after PAF injury. During this process, the voltage-gated sodium channels (VGSCs) and voltage-gated calcium channels (VGCCs) are both enhanced, which have played key roles in releasing neurotransmitters from the nociceptors (Field, Hughes et al. 2000, Altier and Zamponi 2004). Besides, there are also great changes in the sodium channels; for instance, the expression of Nav 1.3 in the dorsal horn and nociceptive neurons is up-regulated. Meanwhile, Nav 1.8 expression is also up-regulated, which plays a vital role in changing the threshold and forming hyperalgesia. Additionally, other sodium channels are also involved in forming neuropathic pain (Hains, Klein et al. 2003, Wood, Boorman et al. 2004, Luiz and Wood 2016), which together with some potassium channels and calcium channels, have exerted some functions in processing neuropathic pain. Typically, the non-selective cation channel TRPV1, which is vital in the inflammatory pain, is altered after nerve injury. Besides, TRPV1 is up-regulated in the uninjured sensory fibers (Hudson, Bevan et al. 2001, Huang, Zhang et al. 2006,

Khomula, Viatchenko-Karpinski et al. 2013, Orestes, Osuru et al. 2013, Yamamoto, Suzuki et al. 2016, Leo, Schmitt et al. 2017). Simultaneously, other ion channels, like TRPA1, TRPM8 or P2X3, are also partially changed after nerve injury, but whether they can cause neuropathic pain remains unclear so far (Shinoda, Kawashima et al. 2007, Eid, Crown et al. 2008, Xu, Li et al. 2011).

1.2.4. Central sensitization mechanisms of neuropathic pain

Central sensitization is the process that amplifies the incoming signals within the central nervous system (CNS), which can form a long lasting synaptic plasticity from a burst activity of the nociceptors. Moreover, it can also amplify signals from the primary afferents to induce the more severely intensive reaction in postsynaptic neurons known as the hyperalgesia. Through this central sensitization process Typically, both the C fibers and Aβ fibers signals can be amplified through this central sensitization process (Ikoma, Fartasch et al. 2004, Baron, Binder et al. 2010, Ploner, Lee et al. 2011).

Three main mechanisms are involved in the central sensitization process, including glutamatergic neurotransmission alteration (N-methyl-D-aspartate receptor (NMDA receptor)-mediated hypersensitivity), loss of GABAergic or glycinergic inhibition, and neuroglia interactions (Basbaum, Bautista et al. 2009).

Usually, the glutamate receptor cannot be activated only through glutamate binding as a result of the Mg^{2+} blocking of the receptor. However, after the persistent stimulation of peripheral noxious stimulus, the released glutamate participates in the postsynaptic reaction of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor), which opens the Na^{+} and Ca^{2+} channels, rendering the influx of Na+ and Ca2+ and creating the rapid excitatory postsynaptic potentials. These potentials will thereby trigger the VGCCs and lead to a higher level of depolarization (Dickenson, Chapman et al. 1997, Collins, Sigtermans et al. 2010). Finally, the continuous accumulation of depolarization can remove the Mg^{2+} blocking of the NMDA receptor, so that it can be activated by glutamate binding. This will also result in cell

depolarization and induce Ca^{2+} influx, which will thereby enhance signal conduction from nociceptors to spinal dorsal neurons, thus leading to the hyperalgesia reaction .

Increases in excitatory postsynaptic potential and neuronal excitability play a crucial part in neuropathic pain; nonetheless, the loss of dorsal horn inhibition is also very important. To be specific, the inhibitory gamma-amino-butyric-acid (GABA) and glycinergic interneurons in the superficial layers of the dorsal horn can be activated by the nociceptive pain signals, which can thus generate the inhibitory impulse to maintain the inhibition state. GABA, the major inhibitory neurotransmitter in the superficial layer of the dorsal horn, is constituted by $GABA_A$-receptors and $GABA_B$-receptors. Of them, $GABA_A$-receptors serve as the ligand-gated chloride channels(Bhisitkul, Kocsis et al. 1990), which allow the chloride ions to get into cells when activated and lead to inhibitory function. In contrast, $GABA_B$-receptors are the G protein-coupled receptors (GPCRs), which can inhibit the release of both glutamate and SP. Peripheral nerve injury can cause the decrease in GABA-induced inhibitory function due to the loss of GABAergic interneurons in the dorsal horn(Moore, Kohno et al. 2002, Janssen, Truin et al. 2011, Benarroch 2016). Notably, bicuculline, a $GABA_A$-receptor antagonist, is used in a pharmacological study to induce neuron hyperexcitability and neuropathic pain(Sorkin, Puig et al. 1998). Moreover, another study has promoted the function of GABAergic interneurons through the pharmacological method, which can alleviate neuropathic pain(Gwak and Hulsebosch 2011). Besides, another important inhibitory reaction is related to opioid-receptor, which is also reduced after peripheral nerve injury. This will lead to reduced synthesis and release of Cholecystokinin (CCK), which is responsible for the opioid inhibitory function(Besse, Lombard et al. 1992).

After peripheral nerve injury, many microglia and astrocytes will be accumulated around the injured area, and the activated microglia can therefore affect the ligand-gated ion channel receptors and GPCRs (CB_2 or P2U purinoceptors), leading to the increased intracellular Ca^{2+} and activation of p38 MAPK. In addition, many different signal molecules will be secreted, such as the cytokines (tumor necrosis factor alpha (TNF-α)),

chemokines and trophic factors, which can enhance the sensitization of neuropathic pain (Inoue, Tsuda et al. 2005, Tsuda, Inoue et al. 2005, Wieseler-Frank, Maier et al. 2005).

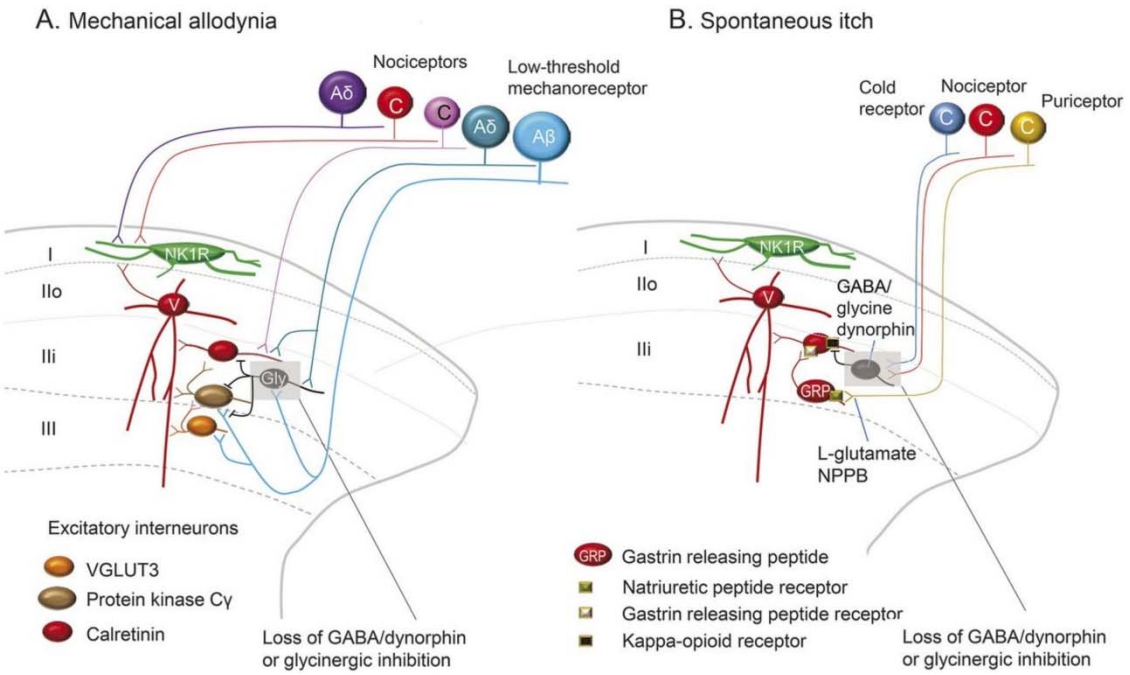

Fig.2. Mechanism of mechanical allodynia and itch. The loss of GABA, glycinergic or dynorphin inhibition in the dorsal horn. (Benarroch 2016)

1.2.5. Stem cell transplantation (SCT) for SCI

Various types of stem cells have been transplanted into the injured spinal cord; for example, olfactory ensheating cells (OECs), mesenchymal stem cells (MSCs), fetal stem cells (FSCs), and induced pluripotent stem cells (iPSCs). Specifically, OECs were first transplanted by Ramon and Nieto in 1994 (Ramon-Cueto and Nieto-Sampedro 1994). OECs can generate nerve regeneration since they have the functions of astrocytes and Schwann cells (Choi and Gladwin 2015). Great outcomes have been attained after transplantation, as shown in many animal studies. Also, some clinical trials have suggested recovery below the injury level (Tabakow, Raisman et al. 2014). However, the culture and transplantation techniques for OECs remain a source of controversy

(Novikova, Lobov et al. 2011). MSCs are the multipotent stromal cells that can differentiate into osteoblasts, chondrocytes and adipocytes under different culture conditions. In addition, MSCs are shown in a study to differentiate into nerve tissue (Deans and Moseley 2000). Noteworthily, many experiments have verified the benefits of MSCs transplantation for SCI animals(Dasari, Veeravalli et al. 2014). FSCs are collected from the embryos, which can differentiate into neurons, astrocytes and oligodendrocytes. FSCs transplantation has been confirmed in studies to alleviate neuropathic pain or even contribute to the regrowth of the injured nerve fibers; however, the underlying mechanisms remains unclear so far (Kadoya, Lu et al. 2016). Takahashi and Yamanaka had found a way to recover the pluripotency from human fibroblasts(Takahashi and Yamanaka 2006). Nowadays, iPSCs can be produced from many different cells in different ways. In some studies, iPSCs are transplanted into the SCI mouse model, which achieve motor function recovery after transplantation (Kobayashi, Okada et al. 2012).

The current study aims to transplant neural stem cells (NSCs) isolated from the rat embryos, but the interventional management is still challenging (Dworkin, O'Connor et al. 2013), and the underlying pathophysiology remains unknown. More recently, dendritic spine remodelling on the second-order wide dynamic range neurons in dorsal horn (Tan and Waxman 2012), synaptogenesis, dendritic sprouting, astrocytes activation (Gwak, Kang et al. 2012), and even dysregulation of potassium channels (Kv3.4) in DRG have been found to be implicated (Ritter, Zemel et al. 2015). Moreover, the main hypothesis of CNPSCI is changed in receptor function and increased neuronal excitability caused by the loss of inhibitory interneurons. Additionally, it is well known that a lesion at any level in both central nervous system (CNS) and peripheral nervous system (PNS) can lead to changes in the descending inhibitory circuits, while GABA is the inhibitory neurotransmitter that plays an important role in CNPSCI (Sivilotti and Woolf 1994, Gwak, Tan et al. 2006, Jiang, Fuller et al. 2016). An electrophysiological research has confirmed the deficits of GABAergic inhibitory neurotransmitters in

CNPSCI (Moore, Kohno et al. 2002). The application of GABA antagonists can lead to hypersensitivity to non-injurious tactile stimuli (Hao, Xu et al. 1994). Moreover, clinical observations have shown that patients with pain after SCI can respond well to GABA-agonists, and some of them are rated as level A in terms of their efficacy against such central pain(Attal, Cruccu et al. 2010).

1.3. Aims of the study

- We wanted to generate neuropathic pain by establishing a hemisection trauma model in the rat.
- It was our hypothesis that the transplantation of GABAergic neuroprogenitor cells into the spinal dorsal horn may alleviate neuropathic pain.

2. Materials and Methods

2.1. Animal model of neuropathic pain

All animal procedures were approved by the Ethics Committee for Animal Research in the State of Baden-Württemberg (Regierungspräsidium Stuttgart), under the protocol number of C5/12.

35 male Sprague-Dawley (SD) rats weighing 240-260g were housed under standard conditions with free access to both food and water, and kept on a 12h/12h dark/light cycle. All rats underwent lateral dorsal hemisection at T13 level with the cutting depth of 1 mm.

2.2. Surgical procedure

All surgeries were performed in a sterile operating room. The rats were intraperitoneally injected with a mixture of ketamine (Ketaset, 7-10mg/100g bodyweight; Parke Davis, Germany) and xylazine (Rompun 1mg/100g bodyweight; Bayer, Germany) for

anesthesia. Then they were kept on a heating pad to maintain their body temperature intraoperatively. The backs of rats were all shaved before surgery. Later, the T13 vertebra was identified through palpation of 13th rib. The vertebrae of rats were fixed using the forceps of the stereotactic apparatus (Stereotaxic apparatus for rat and mouse, SGL M), followed by laminectomy between the T12-13 vertebral segments. Typically, the dorsal spinal blood vessels could be spared by using the operating microscope, and the dorsal root entry zones could be clearly visualized. Surgery in the sham operation group was finished at this step, without inducing any injury to the spinal cord; whereas the dorsal spinal cords of rats in the other groups were hemisected, just from the cranial to the dorsal root entry zone using a No. 11 scalpel blade. Attention should be paid to strictly keep the section depth of 1 mm (Fig.2). Later, muscles and fascia were sutured, and the skin was sutured as well. The whole surgical procedure was performed under standard sterile conditions. During the first week after surgery, rats were housed separately in cages with a thick layer of sawdust and were given analgesics (Metamizole) supplemented in water. The motor function was controlled from the first post-injury day (PID).

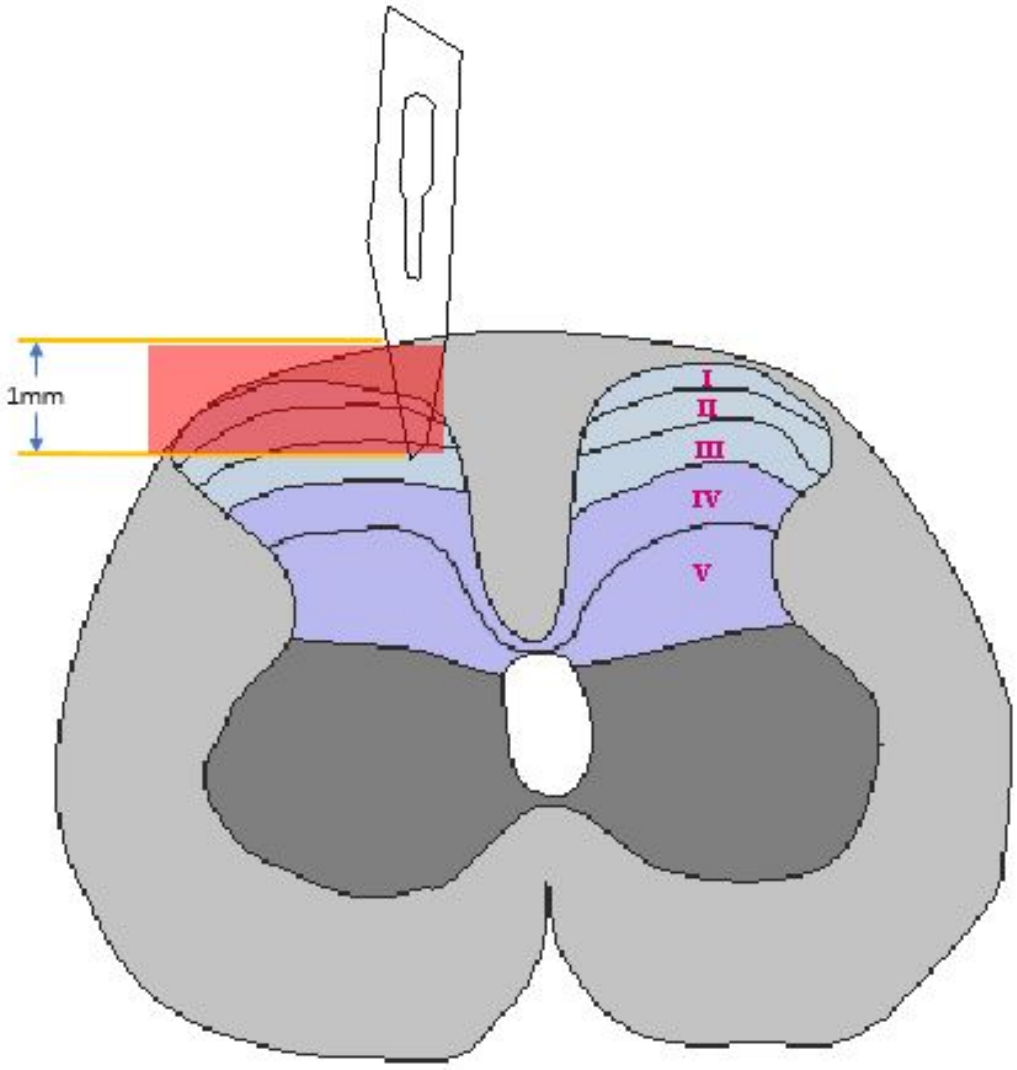

Fig.3. Hemisection of T13 spinal cord using the No. 11 surgical blade at the cutting depth of 1 mm.

2.3. Isolation of neural progenitor cells (NPCs)

Embryonic cortical NPCs were isolated from E14 SD rats. Pregnant rats were intraperitoneally injected with a mixture of ketamine (Ketaset, 7-10mg/100g bodyweight; Parke Davis, Germany) and xylazine (Rompun 1mg/100g bodyweight; Bayer, Germany) for deep anesthesia. Afterwards, a midline ventral incision was made to expose the embryos. Typically, most GABAergic inhibitory interneurons were derived from the ventral telencephalon; to be more accurate, they came from the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and the embryonic

preoptic area (POA). These embryonic brain tissues were collected under the microscope (Fig.3) and placed into a 15 ml conical tube containing cold Hank's balanced salt solution (HBSS; Invitrogen/Gibco). The brain sections were dissected, followed by a low-speed centrifuge at 200 g for 1 min at room temperature (20–25 °C). The supernatant was removed using the MACS neural tissue dissociation kit (Miltenyi Biotec). Then, enzymes were added step by step to each 15 ml conical tube containing the tissue bulks. A fire-polished glass pipette was pre-wet to triturate the tissue through pipetting up and down for 10–20 times until no tissue bulk was observed. 8 ml N2 medium was then added into each tube to dilute the enzyme mixture. Subsequently, cells were pelleted at 200 g for 5 min at room temperature, and the conical tubes were placed onto the MACSmix tube rotator for 20 min of rotation at room temperature. The tissues samples were then resuspended and plated in N2 growth media (DMEM/F12+N2 supplement, pH7.2; Invitrogen/Gibco) containing the standard concentration of FGF-2 (10ng/ml, FGF-2; PeproTech) at 37˚C (Fig.4).

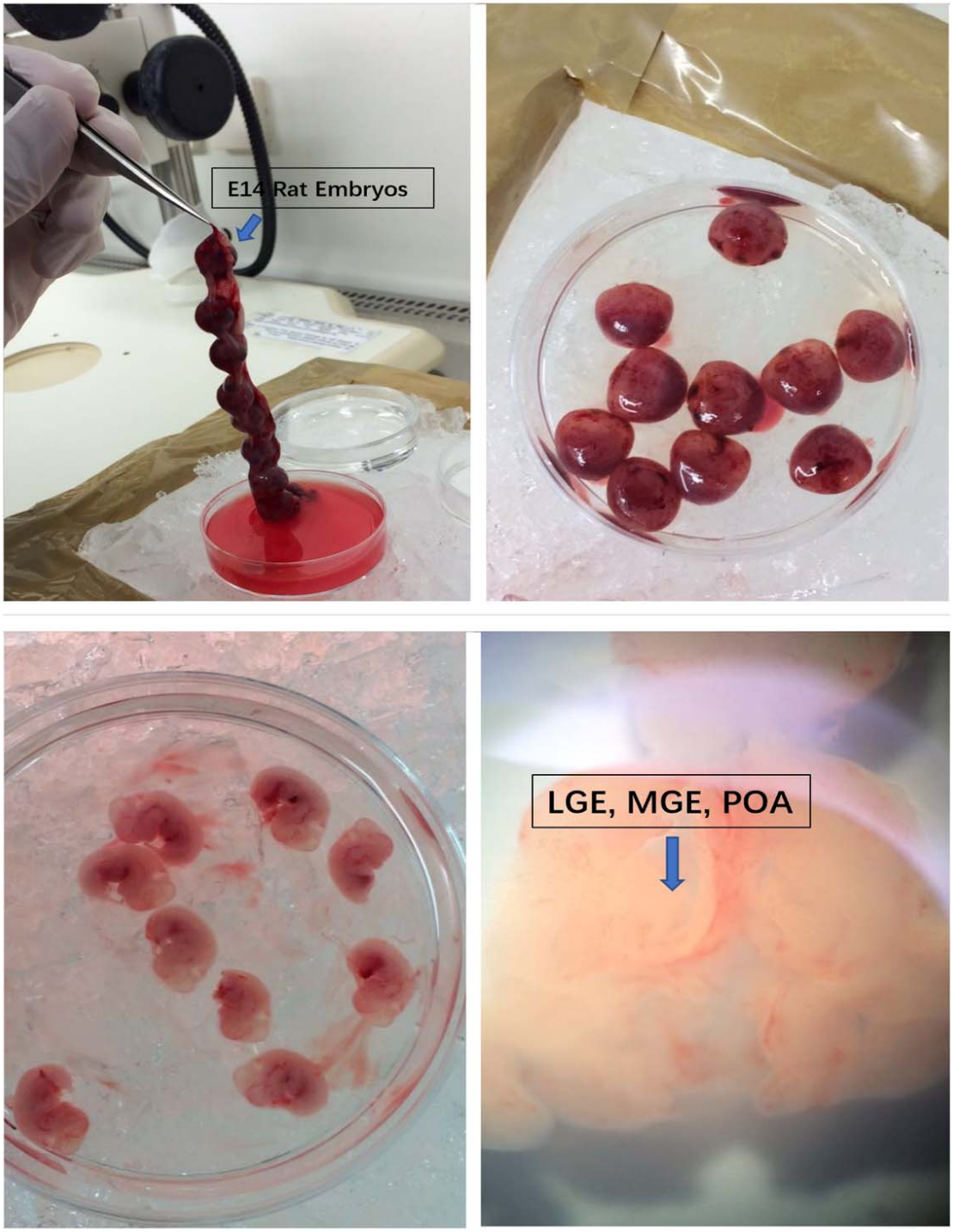

Fig.4. Collection of LGE, MGE and POA tissues from the E14 rat embryos.

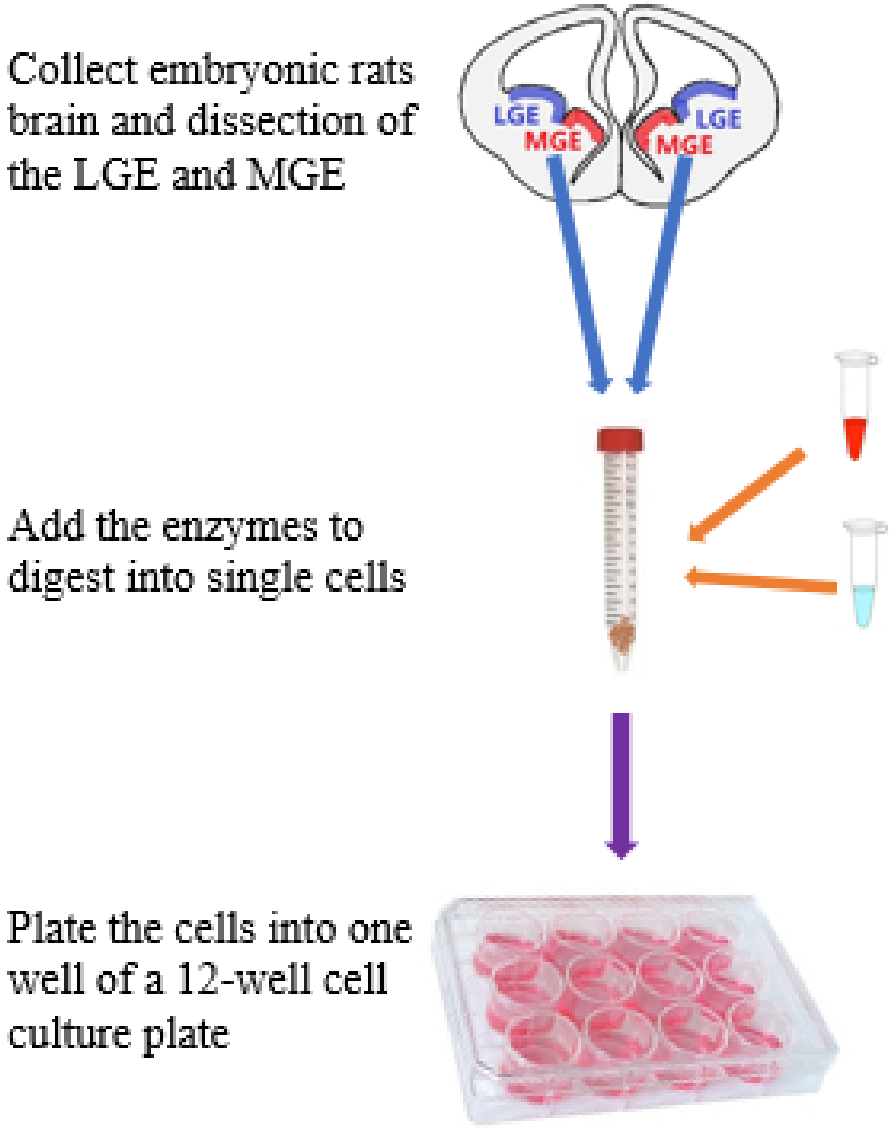

Fig.5. A schematic overview of the major steps in this protocol.

2.4. Differentiation of NPCs

After 7–14 days of culture (Fig.5), all primary spheres were collected without disturbing the attached cells, followed by centrifugation at 200 g for 5 min at room temperature and resuspension in N2 medium supplemented with a final concentration of 1 μM

Retinoic acid (RA; Sigma-Aldrich) and 1 μM Forskolin (FSK; Sigma-Aldrich). Cells were further incubated, and the medium was replaced every day for 4 days continuously. All neurospheres were then collected and digested into single cells, and the viable cells were counted by trypan blue exclusion.

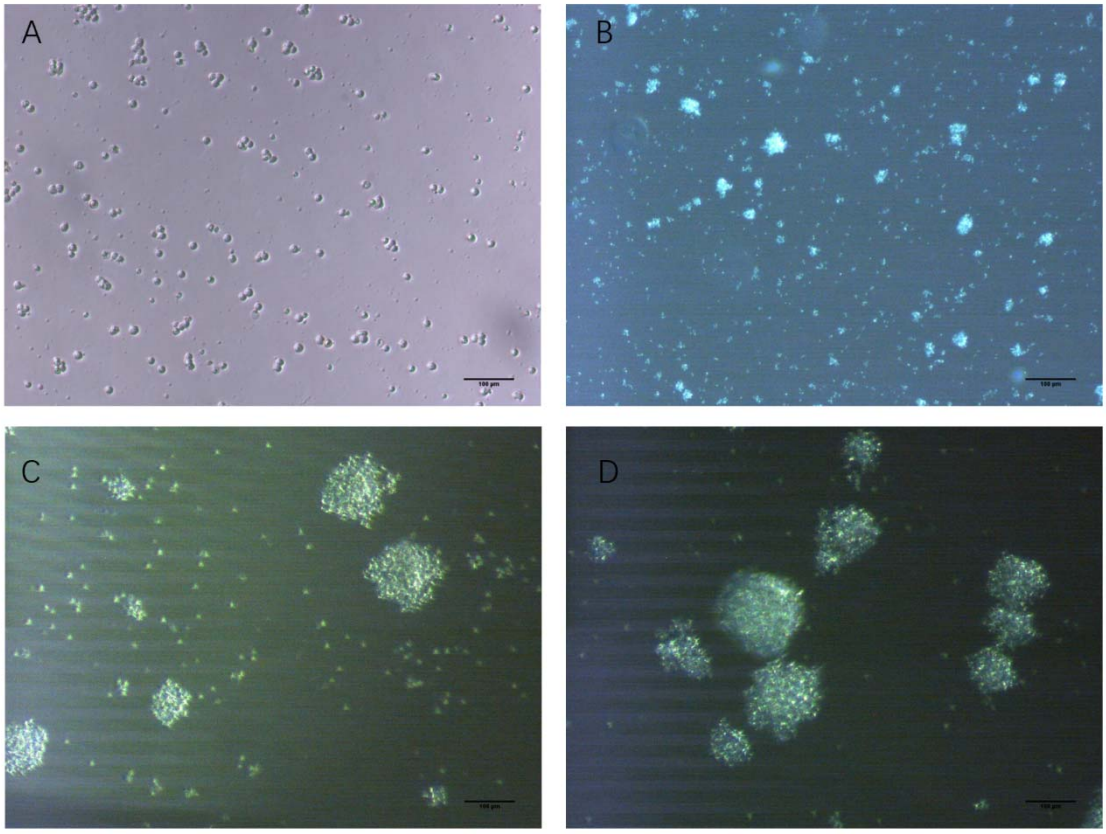

Fig.6. Culture of NPCs. (A) First day of culture. (B) Four days of culture. (C) Eight days of culture (D) Twelve days of culture. All the scale bars were 100um.

2.5. Transplantation of GABAergic NPCs

Only rats developing mechanical neuropathic pain had received cell transplantation (n=15). The animals were anesthetized and a T13–L1 laminectomy was performed aseptically to expose the L3–L4 lumbar spinal cord. Cells were loaded into the Hamilton microinjection syringe (PN 65460-06, gauge 33, OD=0.21 mm x ID= 0.11, Point style 4 comes with 12º; Hamilton), and injected into the hemi-section side, with the syringe being placed 0.5 mm from the dorsal central vein at the injection depth of

0.5 mm to 1 mm. Three injections of 4ul NPCs (5×104 cells/μl) were applied to the experiment group, while PBS was injected in the control group (n=15), and the injection speed was controlled by a micro injection pump at 1μl/min. Afterwards, the syringe was left in place for 1 min to prevent cell backflow (Fig.6). Muscles were later sutured lay-by-layer to cover the injection site, and the skin was closed with wound clips. All rats were placed in some heated cages for recovery after surgery and received immunosuppression with cyclosporine A (i.p.,10mg/kg; Bedford Labs, OH, USA) from one day prior to transplantation until sacrifice.

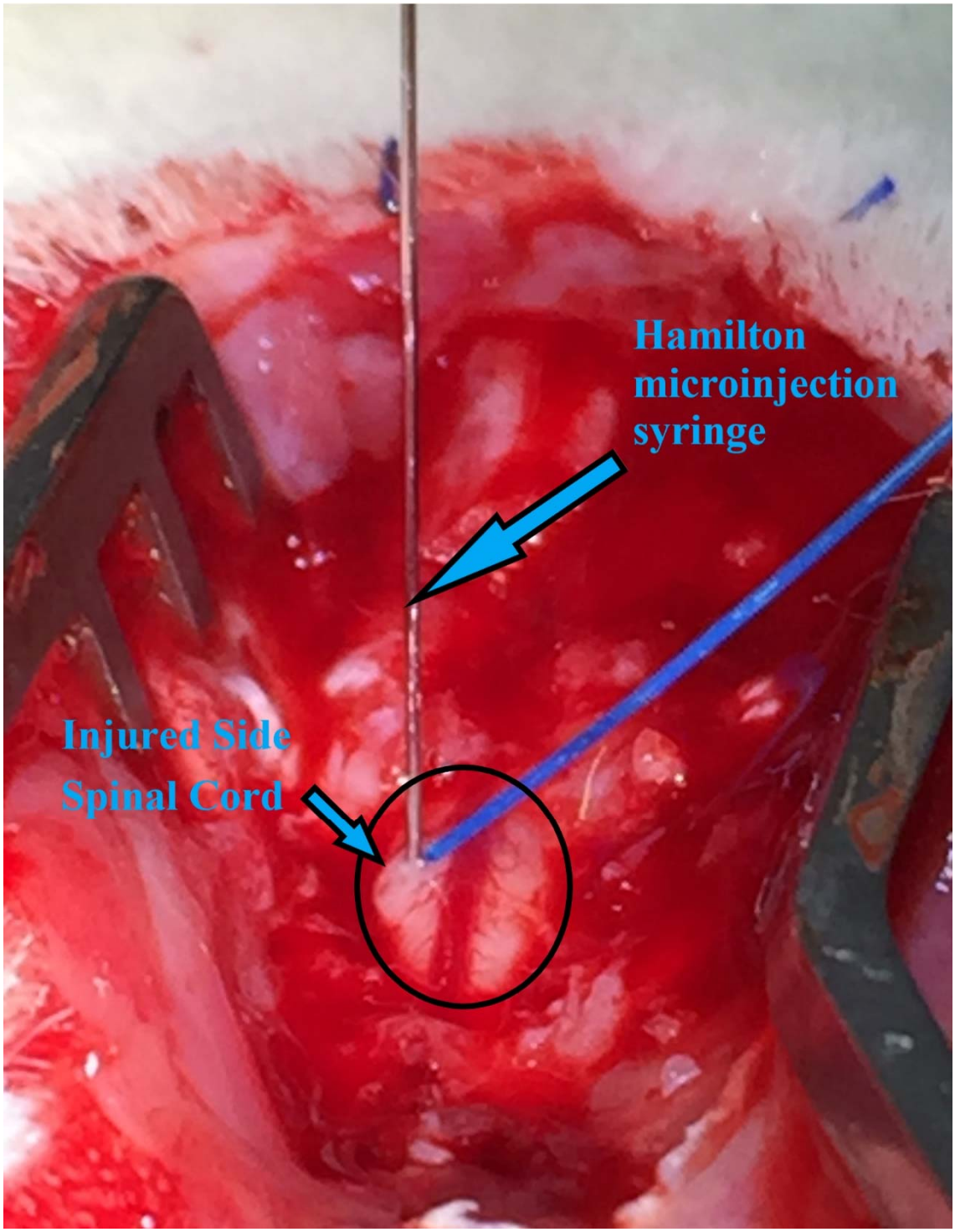

Fig.7. Transplantation of cells and PBS.

2.6. Motor function assessment

To confirm the preservation of postoperative locomotor function, the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale was used (Basso, Beattie et al. 1995). A round plastic pool 100 cm in diameter and 17 cm in height was used in our experiment to monitor the motor function in rats. Then, the ipsilateral hind limb on the injury side and contralateral hind limb were both evaluated from the first day, and weekly after the surgery. Moreover, videos of the evaluation were also taken each time. Each rat was examined by two examiners at the same time, so as to minimize the observer-related bias. The BBB scale, which is sensitive to the animals with mild SCI, is a semiquantitative scale with the values ranging from 0 to 21. The scoring depends on the following forms (Table 2) regarding the limb movement, trunk position, abdomen, paw placement, stepping, coordination, clear toe, predominant paw position, trunk instability and tail. The rating scales were shown in Table 3. In the scale table, 0 suggested no movement of the hindlimb, whereas 21 indicated full function of plantar stepping, coordinated gait, toes movement, trunk stability and tail elevation.

Table 2. BBB locomotor rating sheet.

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 scale.(Basso, Beattie et al. 1995)

I. Early stage of recovery (hindlimb joint movements)
0. No observable hindlimb (HL) movement
1. Slight movement of one or two joints, usually the hip and/or knee
2. Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
3. Extensive movement of two joints
4. Slight movement of all three joints of the HL (hip, knee, and ankle)
5. Slight movement of two joints and extensive movement of the third
6. Extensive movement of two joints and slight movement of the third
7. Extensive movement of all three joints of the HL
II. Intermediate stage of recovery (coordination in stepping ability)
8. Sweeping with no weight support or plantar placement of the paw with no weight support
9. Plantar placement of the paw with weight support in stance only (i.e., when stationary) or occasional, frequent, or consistent weight supported dorsal stepping and no plantar stepping
10. Occasional weight supported plantar steps, no FL-HL (for ellimb-hindlimb) coordination
11. Frequent to consistent weight supported plantar steps and no FL-HL coordination
12. Frequent to consistent weight supported plantar steps and occasional FL-HL coordination
13. Consistent weight supported plantar steps and frequent FL-HL coordination
III. Late stage of recovery (details, refinement of locomotion)
14. Consistent weight supported plantar steps, consistent FL-HL coordination, and predominant paw positions during locomotion are rotated or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping
15. Consistent FL-HL coordination and no toe clearance or occasional toe clearance during forward limb advancement. Predominant paw position is parallel to the body at initial contact
16. Consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement. Predominant paw position is parallel at initial contact and rotated at lift off
17. Consistent FL-HL coordination during gait and toe clearance occurs frequently during forward limb advancement. Predominant paw position is parallel at initial contact and at lift off
18. Consistent FL-HL coordination during gait and toe clearance occurs consistently during forward limb advancement. Predominant paw position is parallel at initial contact and rotated at lift off
19. Consistent FL-HL coordination during gait and toe clearance occurs consistently during forward limb advancement. Predominant paw position is parallel at initial contact and lift off Tail is down part or all of the time
20. Consistent coordinated gait; consistent toe clearance. Predominant paw position is parallel at initial contact and lift off, but there is trunk instability. The tail is consistently up
21. Coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, and tail is consistently up

2.7. Pain assessment

2.7.1. Mechanical pain

The mechanical thresholds on the plantar surface of the rats were determined using the von Frey filaments (Dynamic Plantar Aesthesiometer, Cat. No. 37400-001, Ugo Basile, Italy) before and after surgery. Typically, all rats were trained every day for one week

before surgery. Initially, they were kept in a quiet testing environment for 30 minutes in order to adapt to the surrounding, before they were put into a transparent plastic animal enclosure over a perforated platform (Cat. No. 37450-278, Ugo Basile, Italy) for another 20 minutes. To apply the mechanical stimulation, the central part of the hind paw was targeted under the guidance of a mirror. Afterwards, a dynamic force ranging from 0 to 50 grams was applied within a time frame of 20 seconds to the target point through a metal filament. Specifically, the force of 50 grams was set as the maximal cut-off force, so as to protect the paw from a stab injury. Concretely, the pain threshold of the mechanical stimulation was recorded through two different methods, namely, effective hind paw withdrawal reflex and other behavioral signs of pain. Both left and right hind paws were tested for five times to calculate the mean of the mechanical force, and the baseline value for the mechanical threshold was set one day before surgery.

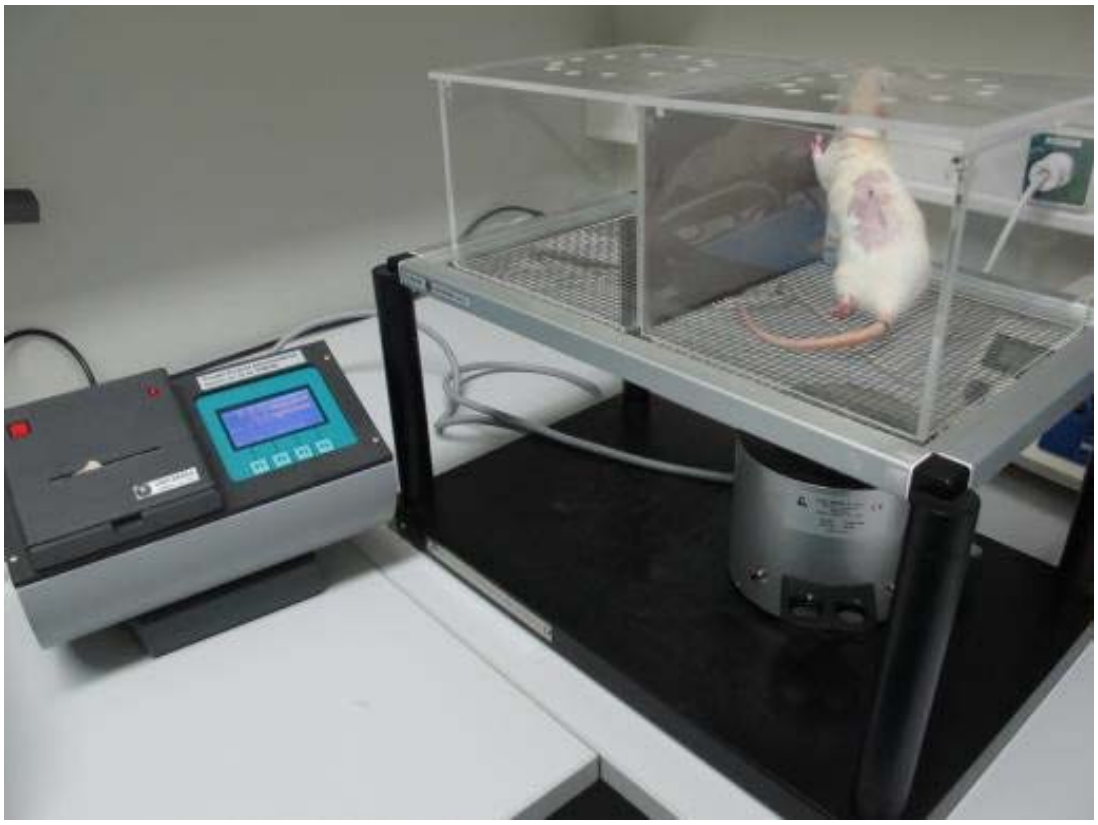

Fig.8. Pain assessment electronic von Frey filaments for quantification of tactile sensation (thickly-myelinated fibers) (Ugo Basile, Italy)

2.8. Evaluation and statistical analysis

All data were analyzed using the Graph Pad Prism 7.0 software (Graph-Pad Software Inc., San Diego, CA, USA). For all statistical analyses, a difference of $P<0.05$ was estimated as statistically significant.

3. Results

In this project, the GABAergic precursors are transplanted into the first relay station of the pain pathway to inhibit pain transmission, using the dorsal spinal cord hemi-section rat model. The rats used in our study are divided into three subgroups, including shame operation group, transplanted with culture medium group and transplanted with GABAergic precursors group. Each group have 15 rats.

3.1. Motor function

After the first day of surgery, the BBB score on the injury side of Sham Operation Group was 19.53 ± 1.19 (Fig.8, Table 4), which was almost the same as the normal score, suggesting that rats had consistent plantar stepping and consistent coordinated gait. Three days later, the BBB score recovered fully to 21. On the first day after hemi-section surgery, the BBB score on the injury side of Transplant PBS Group was 10.867 ± 2.560, which recovered to 18.667 ± 2.610 after the first week, decreased to 14.867 ± 3.441 after transplantation, and recovered to normal level in the second week after transplantation of PBS (Fig.9, Table 5). The BBB score on the injury side of Transplantation of Cells Group on the first day of hemi-section surgery was 12.267 ± 2.052, which resumed to 19.867 ± 1.959 seven days later, revealing a nearly complete recovery. This score also indicated the facility of extension movements of all joints, bearing of body weight and recovery of coordination between the hindlimbs and forelimbs. After cell transplantation, such score decreased to 16.333 ± 2.093, and the motor function had markedly recovered. One week later, the motor score was 20.867 ± 0.516, which indicated the rats had consistent trunk stability and could easily step (Fig.10, Table 6). The results of BBB scores among the three groups were compared, as

shown in Fig.11. Most importantly, no rat showed a sign of bladder or bowel dysfunction during the two surgeries.

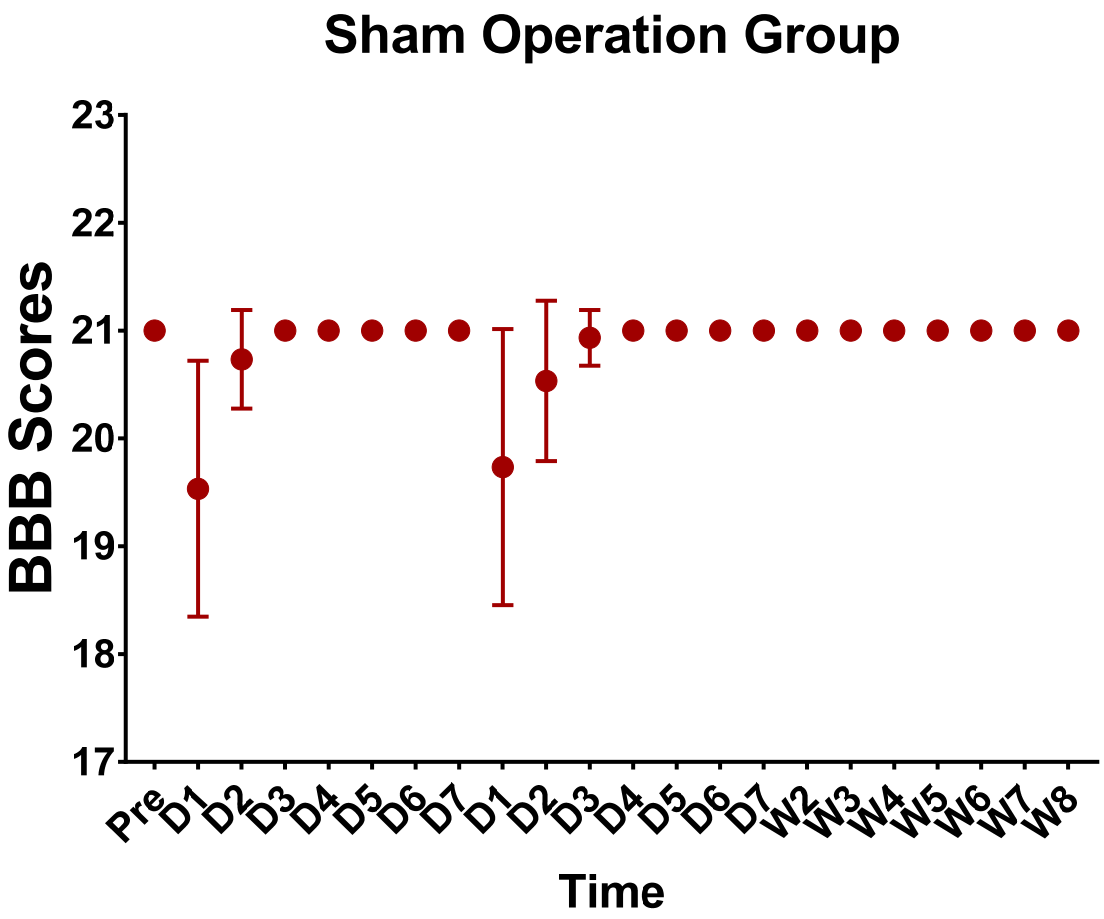

Fig.9. The BBB scores of the sham operation group. The locomotor function of this group showed only slight difference within the normal level. The BBB score was 19.533 ± 1.187 on the first day after sham SCI operation, while that on the first day after sham transplantation operation was 19.733 ± 1.280.

Table 4. Quantification of BBB Scores in sham operation group.

	Mean	SD	N
Pre SCI	21.000	0.000	15
D1 after SCI	19.533	1.187	15
D2 after SCI	20.733	0.458	15
D3 after SCI	21.000	0.000	15
D4 after SCI	21.000	0.000	15
D5 after SCI	21.000	0.000	15
D6 after SCI	21.000	0.000	15
D7 after SCI	21.000	0.000	15
D1 after Trans.	19.733	1.280	15
D2 after Trans.	20.533	0.743	15
D3 after Trans.	20.933	0.258	15
D4 after Trans.	21.000	0.000	15
D5 after Trans.	21.000	0.000	15
D6 after Trans.	21.000	0.000	15
D7 after Trans.	21.000	0.000	15
W2 after Trans.	21.000	0.000	15
W3 after Trans.	21.000	0.000	15
W4 after Trans.	21.000	0.000	15
W5 after Trans.	21.000	0.000	15
W6 after Trans.	21.000	0.000	15
W7 after Trans.	21.000	0.000	15
W8 after Trans.	21.000	0.000	15

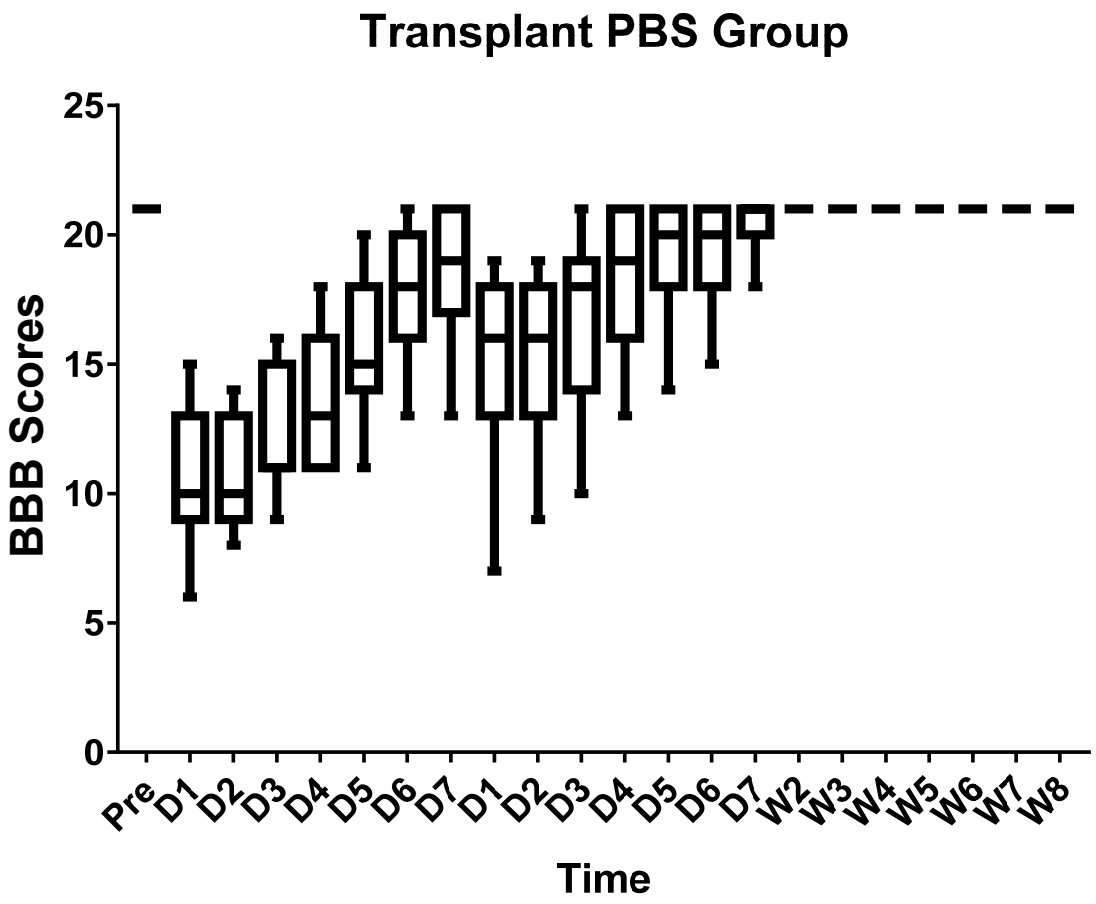

Fig.10. The BBB scores in PBS transplantation group. The BBB score was 10.867 ± 2.560 on the first day after SCI operation, which had recovered to 18.667 ± 2.610 one week later and was decreased to 14.867 ± 3.441 after transplantation. It had recovered to normal level in the second week after transplantation.

Table 5. Quantification of BBB Scores in PBS transplantation group.

	Mean	SD	N
Pre SCI	21.000	0.000	15
D1 after SCI	10.867	2.560	15
D2 after SCI	10.800	2.145	15
D3 after SCI	12.400	2.324	15
D4 after SCI	13.867	2.532	15
D5 after SCI	15.600	2.823	15
D6 after SCI	17.667	2.690	15
D7 after SCI	18.667	2.610	15
D1 after Trans.	14.867	3.441	15
D2 after Trans.	15.333	3.039	15
D3 after Trans.	16.667	3.309	15
D4 after Trans.	18.067	2.815	15
D5 after Trans.	19.133	2.416	15
D6 after Trans.	19.600	1.844	15
D7 after Trans.	20.400	1.056	15
W2 after Trans.	21.000	0.000	15
W3 after Trans.	21.000	0.000	15
W4 after Trans.	21.000	0.000	15
W5 after Trans.	21.000	0.000	15
W6 after Trans.	21.000	0.000	15
W7 after Trans.	21.000	0.000	15
W8 after Trans.	21.000	0.000	15

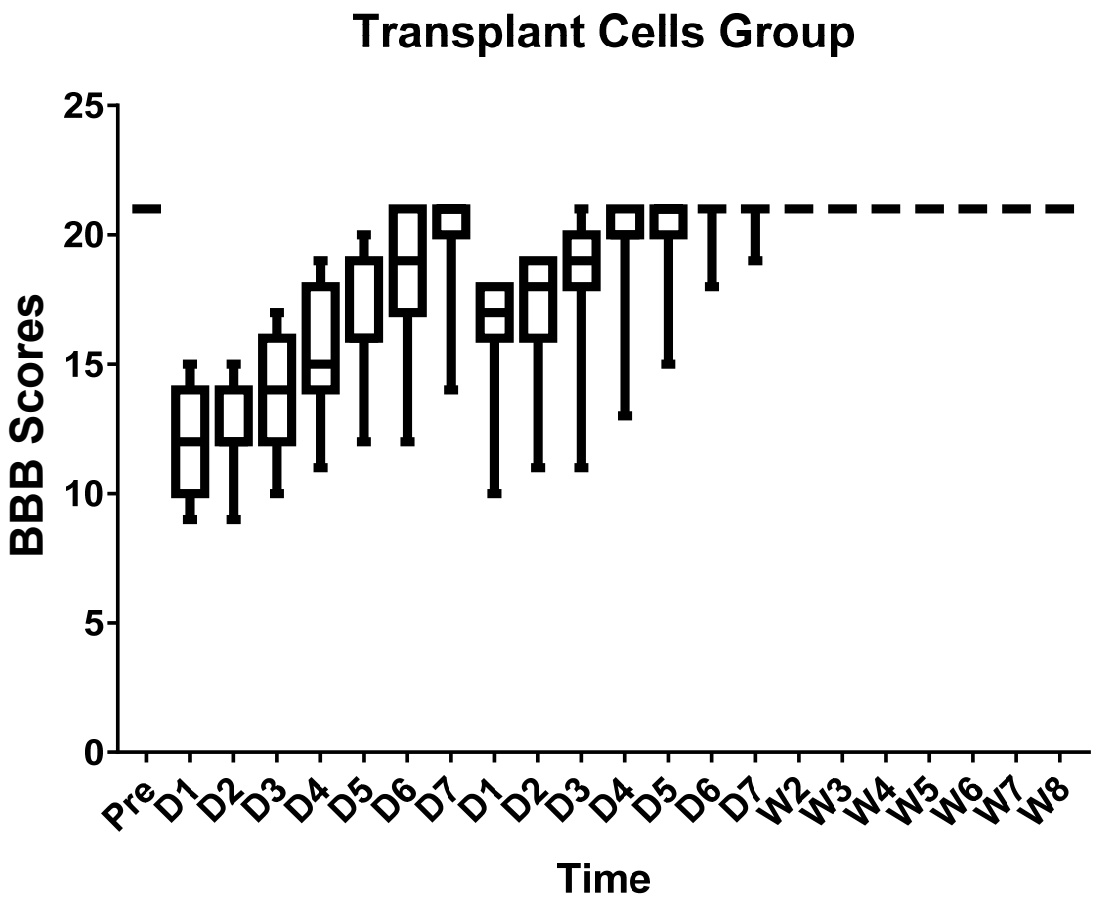

Fig.11. BBB scores in cells transplantation group. The BBB score on the injury side on the first day of hemi-section surgery was 12.267 ± 2.052, which had resumed to 19.867 ± 1.959 seven days later and was decreased to 16.333 ± 2.093 after cell transplantation. The motor function had markedly recovered after transplantation, and the motor score was 20.867 ± 0.516 one week later.

Table 6. Quantification of BBB Scores in cells transplantation group.

	Mean	SD	N
Pre SCI	21.000	0.000	15
D1 after SCI	12.267	2.052	15
D2 after SCI	12.600	1.805	15
D3 after SCI	13.933	2.251	15
D4 after SCI	15.467	2.446	15
D5 after SCI	16.933	2.434	15
D6 after SCI	18.400	2.558	15
D7 after SCI	19.867	1.959	15
D1 after Trans.	16.333	2.093	15
D2 after Trans.	17.200	2.178	15
D3 after Trans.	18.667	2.469	15
D4 after Trans.	19.867	2.031	15
D5 after Trans.	20.400	1.549	15
D6 after Trans.	20.733	0.799	15
D7 after Trans.	20.867	0.516	15
W2 after Trans.	21.000	0.000	15
W3 after Trans.	21.000	0.000	15
W4 after Trans.	21.000	0.000	15
W5 after Trans.	21.000	0.000	15
W6 after Trans.	21.000	0.000	15
W7 after Trans.	21.000	0.000	15
W8 after Trans.	21.000	0.000	15

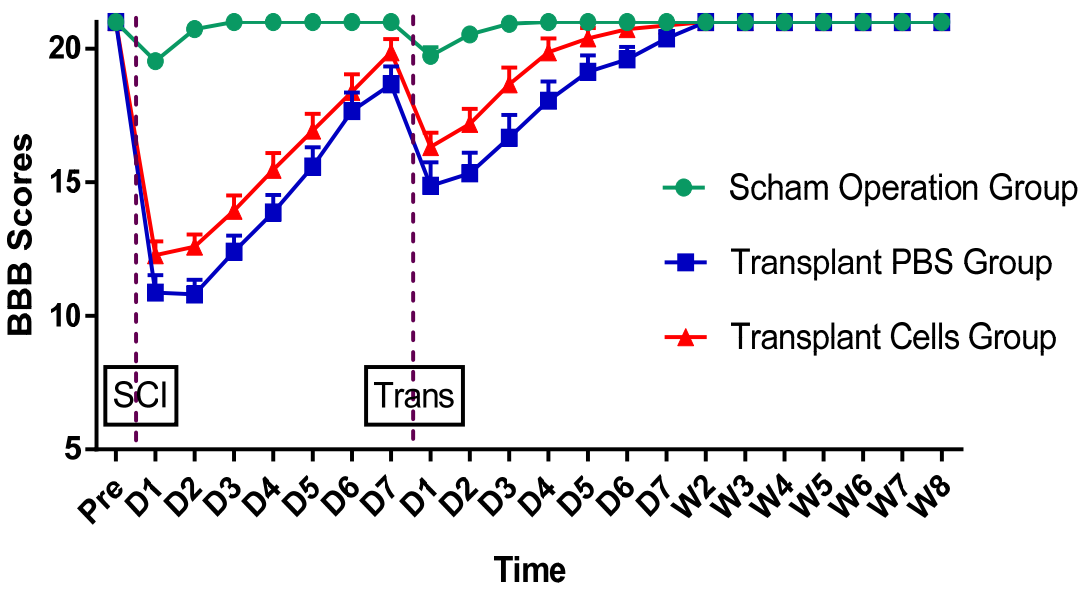

Fig.12. BBB Score on the injury (dorsal hemisection) side of the hindlimbs. The X-axis indicated the time from the first week after SCI measured in days to the first week after transplantation measured in days and then weekly. The motor function was recorded daily for one more week. The BBB scores were measured weekly one week later until the eighth week. "Pre" indicated before surgery. The BBB scores in hindlimbs were 21 before SCI and in W2, W3, W4, W5, W6, W7, and W8, suggesting complete recovery of the motor function.

3.2. Mechanical allodynia

According to the report by Jutatip Guptarak et al., a 23% natural variation of mechanical thresholds was observed in naive rats (Sivilotti and Woolf 1994). In our study, the threshold of mechanical allodynia was found to be decreased by more than 25% (Fig. 2, red and green lines).

At the assessment one week after SCI, a substantial decrease in the mechanical pain threshold of the hindlimb on the injury side was noted in Transplant PBS Group (32.578 ± 2.700 g, Table. 7) and Transplant Cells Group (28.357 ± 1.307 g, Table 6), indicating mechanical allodynia. More importantly, during the whole eight-week evaluation period, no significant spontaneous recovery of the mechanical pain threshold was observed (Fig.15). On the contralateral side, no mechanical allodynia had been observed

throughout the whole evaluation period. Typically, the mechanical pain was slightly alleviated after GABAergic stem cell transplantation, which remained lower than the mechanical allodynia threshold level.

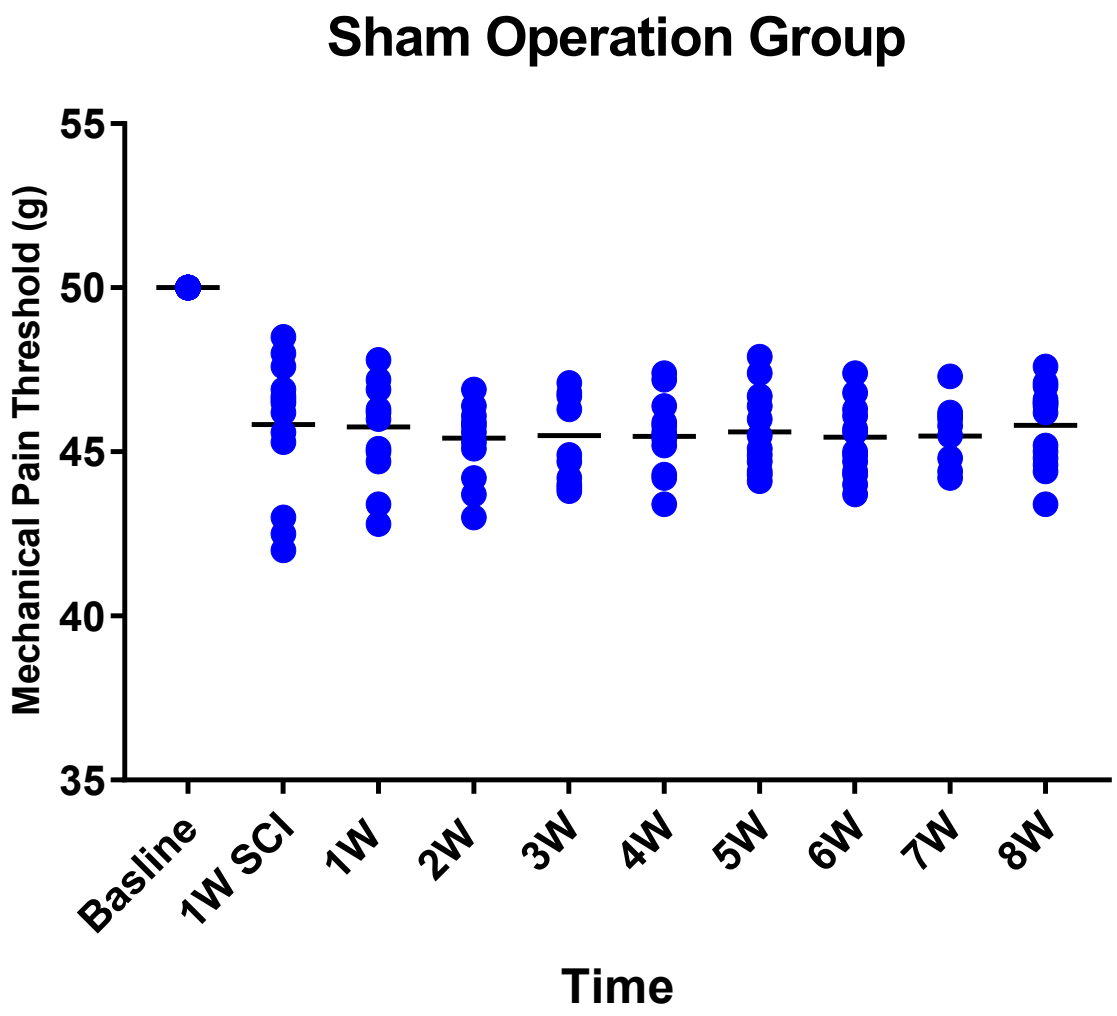

Fig.13. Mechanical pain threshold in the sham operation group. The baseline was set at 50 g. The mechanical pain thresholds on the injury side hind paw were all around 45 g. The quantitative results were shown in Table 7. X-axis was the Time, while Y-axis represented the Mechanical Pain Threshold (g). Mean ± SD.

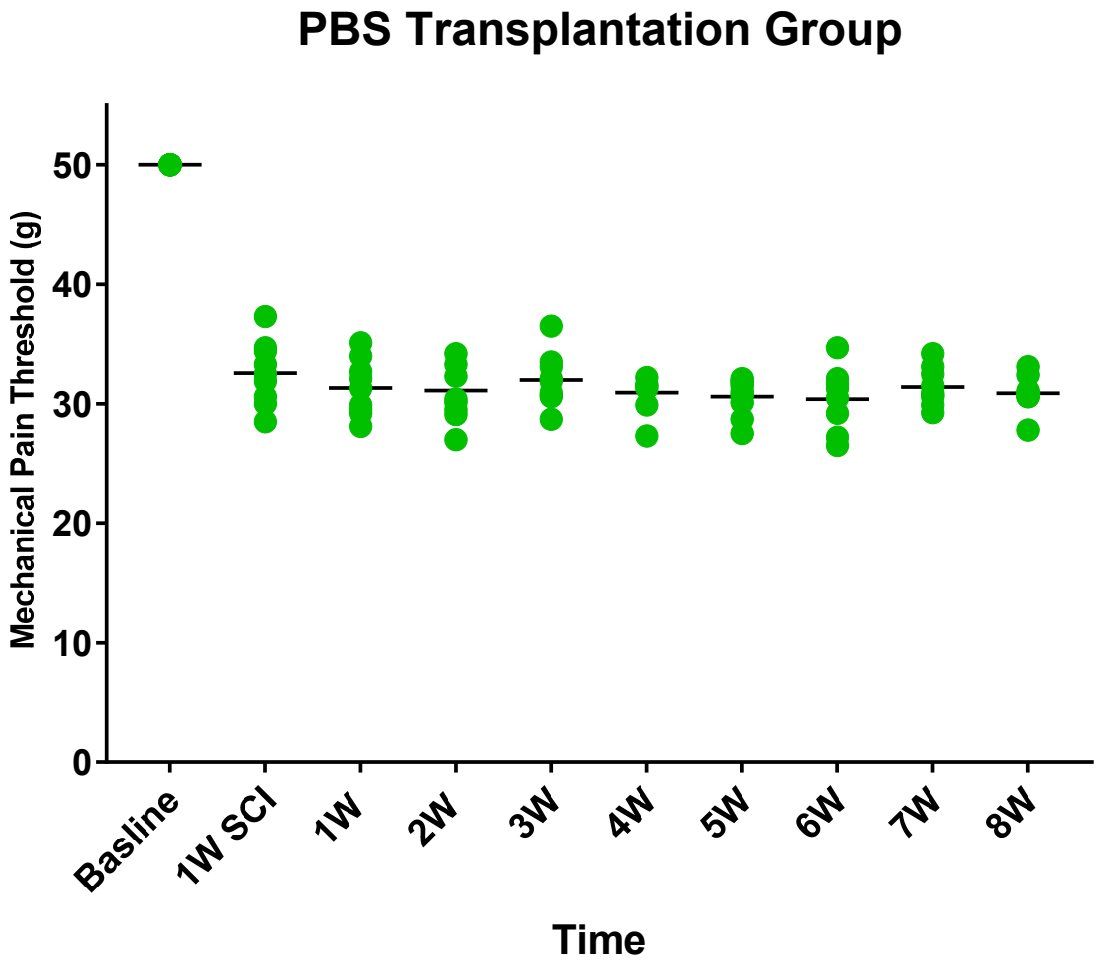

Fig.14. Mechanical pain threshold in PBS transplantation group. The baseline was set at 50 g. One week after SCI surgery, the mechanical pain threshold on the injury side hind paws was decreased to 32.578 ± 2.700, and no substantial change was observed during the next several weeks. The quantitative results were presented in Table 6. X-axis represented the Time, while Y-axis stood for the Mechanical Pain Threshold (g). Mean ± SD.

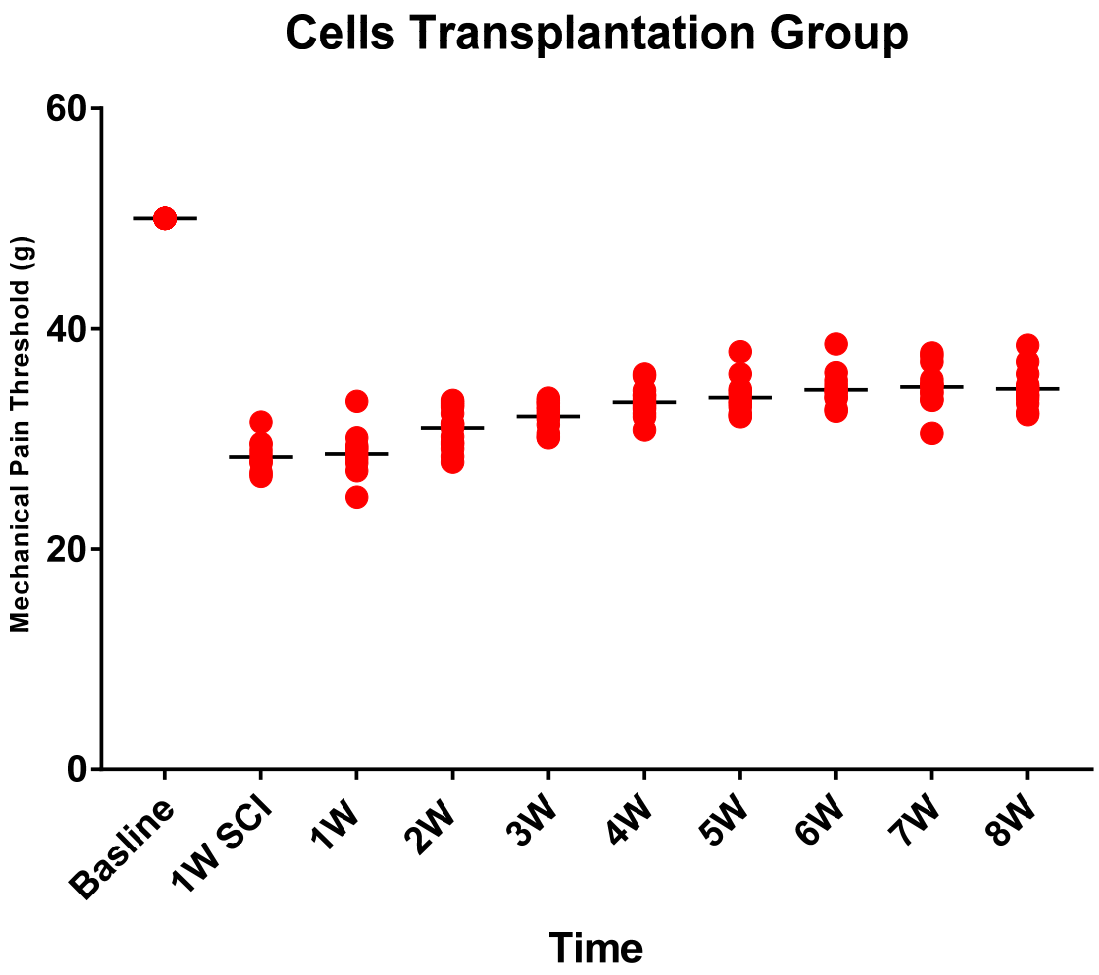

Fig.15. Mechanical pain threshold in cells transplantation group. The baseline was set at 50 g. One week after SCI, the mechanical pain threshold was decreased to 28.357 ± 1.307, which had slightly recovered after transplantation of GABAergic progenitor cells. The quantitative results were in Table 6. X-axis was the Time, whereas Y-axis indicated the Mechanical Pain Threshold (g). Mean ± SD.

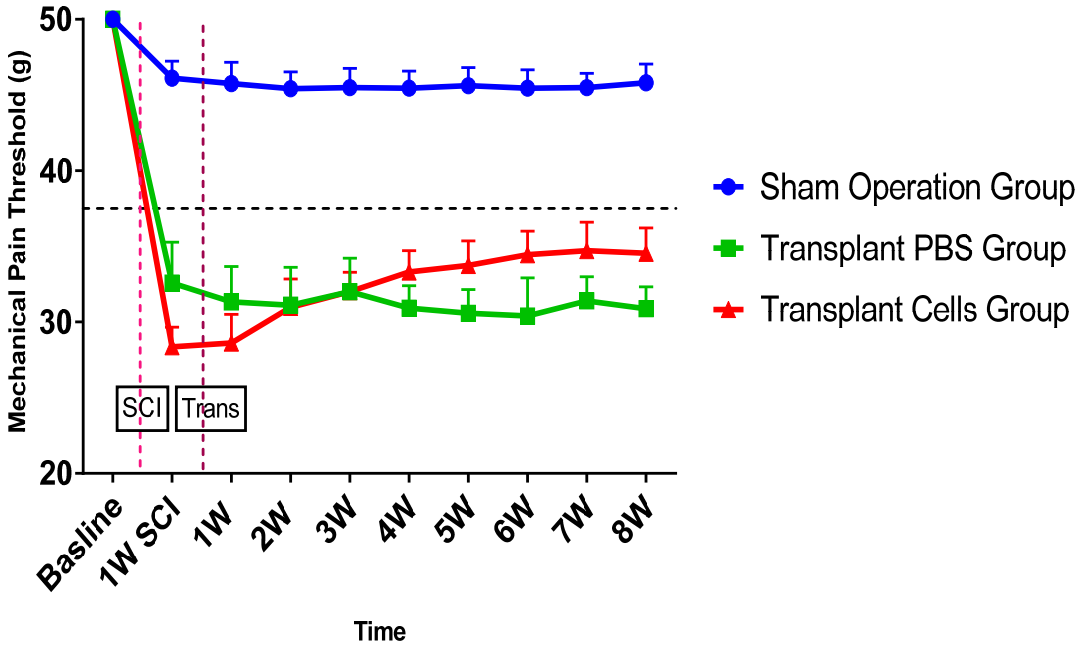

Fig.16. Changes in mechanical pain thresholds following hemisection of the dorsal column. Notably, mechanical allodynia was established ipsilateral to the lesion side as soon as one week after the first operation and one week after transplantation until the eighth week. Results of the three groups were presented, including the Sham Operation Group (blue), Transplant PBS Group (green) and Transplant Cells Group (red). X-axis indicated the Time, while Y-axis suggested the Mechanical Pain Threshold (g). Mean ± SD.

Table 7. Quantification of Mechanical Behavioral Assays

	Sham Operation Group	Transplant PBS Group	Transplant Cells Group
Basline	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

4. Discussion

4.1. The animal models of neuropathic pain

Unilateral hemi-section at T13 spinal cord level in rats represents a model of chronic SCI, which is characterized by hyperexcitability of posterior horn neurons, the major cause of mechanical and thermal allodynia in experimental animals. This animal model has been widely used as a model of neuropathic pain, which can be ascribed to its numerous advantages, such as controllable amount of the injured spinal cord nerves, and less post trauma in hemi-section model than that in spinal contusion animal model. Employing this animal model, Hains et al.(Hains, Fullwood et al. 2001, Hains, Johnson et al. 2001, Hains, Johnson et al. 2003) suggested that 28 days after trauma, hemi-section of spinal cord would result in marked hyperexcitability in a wide range of dorsal horn neurons, which were the second order neurons in the afferent pain pathways. Typically, the neuropathic pain can last for a long time in this animal model undergoing hemi-section(Kim, Yoon et al. 2003). Moreover, many other neuropathic pain models

have also been used, including (1) the peripheral nerve injury models, like chronic constriction injury (CCI), spinal nerve ligation (SNL) and caudal trunk resection; (2) the central neuropathic pain models, such as contusive SCI (Allen's Model), and excitotoxic spinal cord injury; (3) the drug-induced neuropathy models, such as vincristine-induced neuropathy and cisplatin induced neuropathy; and (4) the disease-induced neuropathy models, like peripheral diabetic neuropathy (PDN) and cancer pain models. Several SCI models have been proposed before our study, including clip compression, direct contusion, hemi-section, and dorsal hemi-section. This study mainly aims to analyze the pain sensation after SCI; as a result, a model that would minimally interfere with the efficacy of standard behavioral tests developed for pain assessment is required. The spinal cord hemi-section animal model generates stable neuropathic pain after SCI, without many other physical dysfunctions, like locomotor dysfunction and bladder emptying disorders. According to the motor function of rats in our study, all rats after SCI can achieve complete recovery of the motor function. Typically, the motor function is slightly decreased after transplantation, but it can also recover at a very fast speed. Our previous studies using the clip compression model (Lepski, Jannes et al. 2011) suggested that the animals had developed a stable and severe motor dysfunction, with no neuropathic pain. The contusion model is an accepted and well-established method; however, it may also cause severe and bilateral motor deficits, which may potentially interfere with the pain evaluation. This hemi-section animal model can closely mimic the myelopathic pain presented by patients after spinal cord trauma. Accordingly, the least amount of injury required to produce pain without causing significant motor impairment is determined in this study first of all. All rats after hemi-section of the dorsal horn have decreased mechanical pain threshold. Therefore, the spinal cord hemi-section model seems to be more reliable for the generation of neuropathic pain and its later assessment.

4.2. Stem cells transplantation after SCI-induced neuropathic pain

It was previously believed that the central nervous system (CNS) was incapable of self-regeneration, as illustrated in an extensive review published by Puchala and Windle(Puchala and Windle 1977). These authors had cited the classic animal experiments conducted by the Spanish scientist Santiago Ramón y Cajal in the early twentieth century, who noted that neurons were indeed able to regenerate. Furthermore, he had confirmed that two regenerative processes had taken place following SCI, including (1) creation of nerve ending outgrowths by several axons, and (2) the formation of collateral outgrowths from the preserved axons in the vicinity of the injury in the presence of incomplete spinal transsection. However, none of these processes was able to re-establish effective synapses alone, further contributing to the skepticism on the regeneration ability of the CNS.

More recently, a vast body of evidence has shown that potent factors are able to inhibit neurite growth in adult CNS, particularly in the white matter. Specifically, both oligodendrocyte surface in cell culture and myelin membranes isolated from CNS show strong inhibitory effects on neurite outgrowth (Tatagiba, Brosamle et al. 1997). Besides, many alternative approaches have been proposed aiming to override the hostile environment of white matter in CNS and promote cellular survival as well as axonal regeneration in the injured spinal cord. Meanwhile, many other approaches are available, including the use of pharmacological agents (Houle and Tessler 2003, Baptiste, Tighe et al. 2009), transfection of trophic factor-producing genes (ex vivo gene therapy), or transfection of host tissue with a neurotrophic gene, usually through a viral vector (in vivo gene therapy) (Kwon and Tetzlaff 2001, Blits, Boer et al. 2002). Initially, the ex vivo techniques are based on the implantation of genetically-modified fibroblasts. Typically, the neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), have emerged as important contributors to the regenerative capacity of CNS neurons, rendering them the indispensable components of all successful cellular transplantation strategies (Kwon and Tetzlaff 2001). Notably, the importance of BDNF resides in its

crucial role in tubulin polymerization during dendritic elongation(Yoo, Joung et al. 2007), as well as in the generation and maturation of the newly formed synapses (Babu, Ramirez-Rodriguez et al. 2009). In this study a further crucial role of BDNF has been demonstrated in proper neuronal cell maturation and synapse formation (Babu, Ramirez-Rodriguez et al. 2009). Notably, the use of cellular transplantation technique has represented another highly promising alternative for overcoming the gliotic barrier in the injury site, which may be used to achieve two main goals, namely, (1) regeneration to replace the dead or damaged neurons and induce axonal restoration or plasticity, and (2) repair, the process in which support cells (such as oligodendrocytes) are replaced to promote re-myelination and halt the progressive loss of myelin sheaths. Alternatively, cellular transplants can serve to protect endogenous cells from secondary injury(Eftekharpour, Karimi-Abdolrezaee et al. 2008).

4.2.1. GABAergic progenitor cells compared with other stem cells

Many cell types have been transplanted in the neuropathic pain models, such as OECs, MSCs, FSCs, and iPSCs. Generally, those cells are derived from several different sources, including the internal blastocyst layer (embryonic cells), fetal brain (fetal neural cells), mature neuronal tissue (subventricular zone or dentate gyrus of the hippocampus), or mature non-neuronal tissues (such as bone marrow) (Dasari, Spomar et al. 2007, Conrad, Renninger et al. 2008). Among them, OECs are believed to provide the neurotrophic support through a substrate that can facilitate cellular growth and remyelination in axons spared from injury(Kwon and Tetzlaff 2001). On the other hand, MSCs are abundant even in adult animals, which can be easily obtained from humans by simple iliac crest puncture. These cells are biologically safe, and can eliminate the need for immunosuppression. Typically, MSCs have been suggested to be capable of neuronal differentiation or trans-differentiation(Brazelton, Rossi et al. 2000, Sanchez-Ramos, Song et al. 2000, Mezey, Key et al. 2003). Nevertheless, our previous study suggested that, although MSCs had acquired the morphological and functional

features of neurons, they failed to reach the same maturation stage achieved by neural stem cells derived from human or murine fetuses(Lepski 2012). Unfortunately, such limitation in MSC differentiation remains largely unknown. Based on our previous experience as well as other reports, the role of host microenvironment in proper graft survival and differentiation can not be under-emphasized(Lepski, Jannes et al. 2010). Lu [31] had transplanted MSCs into a chronic SCI rat model, and found that they were genetically modified to express neurotrophin-3 (NT-3), which had given rise to a greater stimulation for axonal growth. However, these strategies would not lead to functional recovery after chronic SCI, indicating that the regenerated axons fail to form the appropriate synapses in the host tissue. Meanwhile, NPCs transfected with Green Fluorescent Protein (GFP) are known to be capable of synaptogenesis when implanted into the hippocampus of adult rats, and the newly formed synapses are predominantly GABAergic. Some researchers also use adult NSCs, which are the tissue-specific somatic cells locating in the periventricular regions and the hippocampal dentate gyrus. NSCs can differentiate into all types of neuronal cells under specific conditions. Specifically, subventricular adult NSCs have been demonstrated in previous work to differentiate into three main types of nerve cells, including neurons, oligodendrocytes and astrocytes(Kim, Auerbach et al. 2002). Additionally, the functional motor recovery has also been demonstrated following transplantation of adult NSCs after pyramidal cortex injury in mice (Lepski, Jannes et al. 2010). Interestingly, our previous studies had observed a strong tendency for the generation of GABAergic neuronal cells from NSCs in a conventional process of differentiation in vitro(Maciaczyk, Singec et al. 2008). GABAergic cells are excitatory during the initial phase of development, which can be attributable to the absence of a chloride and potassium co-transporter. Later, during the maturation process, GABA becomes inhibitory(Ge, Goh et al. 2006). Our studies have determined that, around 60% mature neuronal cells obtained from the differentiation of NSCs in vitro have assumed the GABAergic phenotype(Maciaczyk, Singec et al. 2008). At the same time, changes in GABA expression after SCI or peripheral nerve injury

would lead to inhibitory dysfunction, as mentioned in several studies (Polgar, Gray et al. 2004, Meisner, Marsh et al. 2010). In view of this finding, a hypothesis has been proposed, which is that GABAergic precursor cells may ultimately cause post-synaptic inhibitory potentials, thus reverting the hyper-excitability that develops in the posterior horn neurons after SCI. Therefore, the GABAergic NPCs isolated from MGE of E14 embryo rats are used in this study, with an aim to study the GABA inhibitory function in neuropathic pain after SCI. More GABA or GABAergic cells will be differentiated after transplantation of GABAergic NPCs in the injured area, which will enhance the inhibitory function. Concretely, some studies have been carried out on increasing GABA production to amplify its anticonvulsant effects in epilepsy animal models(Handreck, Backofen-Wehrhahn et al. 2014).

4.2.2. Hypothesis of GABAergic NPCs transplantation in alleviating neuropathic pain after SCI

To recover the inhibitory function of the GABAergic neurotransmitters after SCI, we have transplanted the GABAergic NPCs into the injured spinal cord. Our results suggest that the mechanical threshold is increased one week after transplantation, and has recovered till the end of experiment. Moreover, similar studies have also been carried out. For instance, Mukhida(Mukhida, Mendez et al. 2007) transplanted the mouse striatum GABAergic cells into the neuropathic pain animal models induced by spinal nerve ligation. Moreover, Hendricks(Hendricks, Pak et al. 2006) and Kim(Kim, Jung et al. 2010) had transplanted embryonic stem cells to reduce the SCI-induced neuropathic pain. Additionally, there are various hypotheses regarding the alleviation of neuropathic pain. One of the most popular theory is, that GABA secretion is increased after transplantation. Typically, there are two main points about this theory, which are, that grafted cells can differentiate more GABAergic interneurons(Stubley, Martinez et al. 2001), or they may reduce cell death by releasing trophic factors in order to generate the neuroprotective action (Hollrigel, Toth et al. 1996, Jolkkonen, Halonen et al. 1996).

Besides, some researchers have also mentioned the restoration of the spinal serotonergic system, which can inhibit the pain transmission(Bowker, Westlund et al. 1981, Marlier, Sandillon et al. 1991, Sanchez, Niedbala et al. 1995). The transplanted stem cells can alleviate the neuropathic pain, which may possibly be ascribed to the decreased hyperexcitability in the dorsal horn, as reported by recording the wind-up phenomenon on several studies(Eide, Jorum et al. 1996, Felsby, Nielsen et al. 1996, Sokal and Chapman 2001). Moreover, it has been found in electrophysiological studies that, the post discharge rate is reduced after NPCs transplantation(Drew, Siddall et al. 2004).

4.3. Limitations of the current study

In this study, only the mechanical pain is recorded, which is just one part of the neuropathic pain. Moreover, the thermo-pain threshold should also be monitored to get more proof, and the post-transplant histology is also required in order to detect the transplant survival and cell migration, which will be completed by my colleague.

5. Summary

5.1. Summary

Spinal cord injury (SCI) is a leading cause of permanent disability and chronic pain, which is well-known to induce hyper-excitability in a wide range of neurons in the posterior horn of the spinal cord. In turn, this will be largely responsible for the severe neuropathic pain. Specifically, stem cell therapy represents a promising method for treating neurological deficits stemming from SCIs. However, the key questions still remain to be answered. In this project, the GABAergic precursors are transplanted into the first relay station of the pain pathway to inhibit pain transmission, using the dorsal spinal cord hemi-section rat model. The rats used in our study are divided into three subgroups, including shame operation group, transplanted with culture medium group and transplanted with GABAergic precursors group. Of them, the GABAergic precursor cells transplanted in this study are derived from the telencephalic vesicles from E12 rat

embryos. Moreover, the behaviors of these animals will be assessed in terms of pain sensitivity through applying von Frey filaments on a weekly basis. Two months later, the animals are sacrificed to collect their spinal cords. Then, immunohistochemical staining and analysis under a confocal laser microscope would be performed by my colleagues. The mechanical pain threshold is slightly increased after the transplantation and is not fully restored. The findings of this study suggest that the transplantation of GABAergic precursors can alleviate neuropathic pain caused by a hemi-section of the spinal cord.

5.2. Zusammenfassung

Rückenmarksverletzung (SCI) ist eine führende Ursache für permanente Behinderung und chronischen Schmerz, der bekanntermaßen Überreizbarkeit in einer Vielzahl von Neuronen im Hinterhorn des Rückenmarks induzieren kann. Dies wird wiederum weitgehend für die schweren neuropathischen Schmerzen verantwortlich sein. Insbesondere stellt die Stammzelltherapie eine vielversprechende Methode zur Behandlung von neurologischen Defiziten dar, die von SCIs herrühren. Die Schlüsselfragen müssen jedoch noch beantwortet werden. In diesem Projekt werden die GABAergen Vorläufer in die erste Relaisstation des Schmerzwegs transplantiert, um die Schmerzübertragung zu hemmen, wobei das dorsale Rückenmarkshemisektions Rattenmodell verwendet wird. Die Ratten, die in unserer Studie verwendet wurden, sind in drei Untergruppen unterteilt, einschließlich einer Scham-Operationsgruppe, transplantiert mit einer Kulturmediumgruppe und transplantiert mit einer GABAergen Vorläufergruppe. Von diesen sind die in dieser Studie transplantierten GABAergen Vorläuferzellen von den telenzephalen Vesikeln von E12-Rattenembryonen abgeleitet. Darüber hinaus wird das Verhalten dieser Tiere im Hinblick auf die Schmerzempfindlichkeit durch Anwendung von von Frey-Filamenten wöchentlich untersucht. Zwei Monate später werden die Tiere geopfert, um ihr Rückenmark zu untersuchen. Dann wurden von meiner Kollegin immunhistochemische Färbungen und

Analysen unter einem konfokalen Lasermikroskop durchgeführt. Die mechanische Schmerzschwelle ist nach der Transplantation leicht erhöht und nicht vollständig wiederhergestellt. Die Ergebnisse dieser Studie deuten darauf hin, dass die Transplantation von GABAergen Vorläufern neuropathischen Schmerz lindern kann, der durch eine Hemi-Section des Rückenmarks verursacht wird.

6. List of Figures

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8. Acknowledgements

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9. Erklärung zum Eigenanteil der Dissertationsschrift

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

Die Konzeption der Studie erfolgte in Zusammenarbeit mit Prof. Dr. med. MH Morgalla durchgeführt.

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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 08.08.2018

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