A model of neuropathic pain in brachial plexus avulsion injury and associated spinal glial cell activation

Background: Neuropathic pain is a common and intractable sequel of brachial plexus injury.

Materials and methods: To investigate the underlying mechanisms, we established a unique model of neuropathic pain in rats by creating brachial plexus avulsion injury.

Results: Behavioral test of mechanical stimulation suggested that all rats developed neuropathic pain, and the pain thresholds of bilateral hind limbs significantly decreased. GFAP expression in the cervical spinal cord appeared on day 1 post-injury and increased on day 4. Ionized calcium-binding adaptor molecule 1 expression appeared on day 1 post-injury and increased until day 28. Therefore, the brachial plexus avulsion injury model can imitate the development of neuropathic pain and maintain it.

Conclusion: The activation of astrocyte and microglia in the spinal cord plays a key role in the mechanism and treatment of neuropathic pain.

Keywords: neuropathic pain, new model, astrocyte, microglia

Introduction

Brachial plexus avulsion injury (BPAI) is a most devastating peripheral nerve injury in the upper extremity. When upper extremity suddenly suffer powerful force, especially in traffic accident, all nerve roots from C5 to T1 are completely avulsed from the cervical spinal cord. In this injury, the rootlets of the C5–T1 spinal nerves connecting the central nervous system (CNS) with the peripheral nerves are divided, which makes the whole upper limb paralyzed.1 Obviously, it is impossible to repair by direct repair. These patients are suffering from negative effects of BPAI on sensorimotor functions and quality of life.

In addition to sensorimotor dysfunctions, a number of BPAI patients suffer from neuropathic pain. It is a serious complication, which is long lasting, hard to relieve, and drug resistant. It was reported that patients with BPAI were easier to sustain neuropathic pain than those with other peripheral nerve injuries.2 According to the literature, almost 67% of BPAI patients would develop neuropathic pain.3 Previous studies indicated that neuropathic pain following BPAI is difficult to treat in clinical practice, and most treatments could not relieve the pain effectively.4 Therefore, it is essential to explore its underlying mechanisms, which is not fully understood so far.

Mounting studies have demonstrated the role of spinal glial cell in the occurrence and development of neuropathic pain.5 Garrison et al6 indicated for the first time that the activation of spinal glial cells and astrocyte is associated with hyperalgesia. Paszczuk et al7 also reported that glial cells played an important role in modulating BPAI-induced...
neuropathic pain. Thus, we speculated that glial cells may largely involve in the process of neuropathic pain after BP AI.

In the present study, we established a modified BP Al model in adult rats with neuropathic pain confirmed by behavioral tests. The immunohistochemistry studies of glial fibrillary acidic protein (GFAP; for astrocytes) and ionized calcium-binding adaptor molecule 1 (Iba-1; for microglia) were performed at different post-injury intervals to investigate the change in glial cells in the spinal cord during the process of neuropathic pain formation. This study aimed to establish a modified BP Al model and demonstrate this model can induce neuropathic pain assessing by the activation of spinal glial cell.

Materials and methods

Animals

A total of 60 female adult Sprague Dawley (SD) rats (weight, 180–200 g; age, 6 weeks) were divided into seven groups: one control group (n=12) and six BP AI groups (n=8 per group). Rats were grouped into six BP AI groups by different time points for immunohistochemistry studies as day 1, 4, 7, 14, 21, and 28, respectively. Unlike previous models that are often performed by the anterior cervical approach,7 we designed a modified posterior cervical procedure to produce BP Al.

The procedures were approved by the Animal Research Committee, Fudan University. All experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals as described by the US National Institutes of Health, and all the experiments were designed to minimize both the number of animals used and their suffering.

Surgical procedure of BP Al

The BP Al model was established under anesthesia induced by a 10% chloral hydrate solution injection intraperitoneally. The rat was placed in the prone position. Under sterile conditions, a posterior midline incision was made on the back of the neck. The spinous process of the second thoracic vertebra was found, and the left side muscle was parted to reveal the facet of C4–T1 vertebra. Hemilaminectomy of left C4–T1 was performed by micro-dissecting scissors, and the dorsal roots of C5–T1 were exposed. Then, the dorsal roots of C5–T1 were avulsed from the spinal cord, and the ventral roots were exposed. The ventral roots were also avulsed by the same approach. The surgery was performed under the operating microscope. The key steps were shown in Figure 1.

Sham operation was performed in the control group. Hemilaminectomy of C4–T1 and exposure of brachial plexus nerves (C5, C6, C7, C8, and T1) on the left side were performed in the control group, consistent with that in the experimental group. Then, the incision was closed without brachial plexus avulsion.

Behavioral tests

The behavioral tests were performed in accordance with previous studies.8–11 Before the test, rats were habituated in the testing environment for 30 minutes. All the behavioral tests were carried out blindly. All rats in six BP Al groups were tested at each post-injury time point.

Mechanical pain tests

Mechanical pain was assessed by the method described previously by using Von Frey Filament.8,11 Rats were placed on a metal mesh floor (0.8×0.8 cm² cells) covered by a transparent plastic box that was kept at 30 cm above the floor. After approximately 15 minutes of adaption, we fixed its hind limb with a nipper when the hind limb fell into the grid. The Von Frey filament was used to apply a linearly increasing pressure on the hind limb until the rat withdrew it. The mechanical pain threshold were recorded on days 0 (pre-injury), 1, 4, 7, 1, 21, and 28 post-injury. The stimulating duration was 4 seconds, and the interval between stimulations was 30 seconds. The mechanical pain threshold described as the lowest force that evoked a consistent brisk withdrawal response. The 50% mechanical pain threshold was evaluated and compared with the pre-injury data. The data on day 0 (pre-injury) were set as baseline.

Thermal pain tests

Thermal pain threshold was assessed with thermal pain stimulation system as previously described.8–10 The rat was placed in a glass box with a bottom. The radiant heat source was positioned under the glass floor directly beneath the hind limb. While the beam intensity was set at 5, the time when the rat withdrew the paw was recorded. The thermal pain threshold were also recorded on days 0 (pre-injury), 1, 4, 7, 1, 21, and 28 post-injury. Thermal pain threshold was the duration when rats showed a withdrawal reaction. To avoid heat injury, the machine would be cut off after 25 seconds. Each rat was evaluated five times, and the interval was 3 minutes. The last three thermal pain thresholds were calculated and averaged. The data on day 0 (pre-injury) were set as baseline.

Immunohistochemistry of GFAP (astrocyte) and Iba-1 (microglia)

After behavioral tests at each interval, rats in each BP Al group and two rats in the control group were injected intraperitoneally.
with excessive 10% chloral hydrate solution and then transcardially perfused with 250 mL of 0.9% saline, followed by 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 2 hours. Cervical enlargement was resected, post-fixed in 4% paraformaldehyde for 4 hours, and dehydrated using graded sucrose. The tissue samples were frozen in tissue-freezing medium and sliced into 40 µm transverse sections on a cryostat. Every sixth section was collected and placed in the protective fluid. Approximately 70 sections of the sample were harvested from the rat. All sections were rinsed three times for 10 minutes each in 0.01M PBS (pH 7.4) and then incubated with blocking buffer (10% goat serum [KPL, 71-00-27] and 0.3% Triton X-100 in 0.01 M PBS) overnight for 4°C. Primary antibody, rabbit anti-GFAP (1:1,000; Chemicon Company, Temecula, CA, USA), and rabbit anti-Iba-1 (1:1,000; Chemicon Company) were added to the blocking buffer and incubated with sections at 4°C for 48 hours. The blocking buffer was replaced, and the secondary antibody (fluorescein isothiocyanate [FITC]-conjugated goat-anti-rabbit IgG, 1:2,000; Wuhan Boster Biological Technology, Wuhan, China; BA1105) was added and incubated with sections at 37°C for 2 hours. Activated glial cells were observed with a Leica fluorescence microscope. Five random areas in the spinal dorsal horn of each sample were examined at ×200 magnification. The average number of the activated astrocyte and microglia in these five areas was calculated.

Data analyses
SPSS 15.0 was used for the statistical analyses. These data were presented as mean±SEM. Statistical significance between groups was determined by using one-way ANOVA followed by Student–Newman–Keuls (SNK) test. In the same group, paired sample t-test was used to test the significant difference. P<0.05 was considered as statistically significant.

Results
Behavioral results
The basic behavioral observation
The left forelimb of all rats in the BPAI groups was totally paralyzed. The feeding behavior was not influenced. No evidence of cervical instability was observed after C5–T1 hemilaminectomy. After BPAI, three rats showed self-mutilation behavior of biting the paralyzed limb. The self-damaged area ranged from nails to the elbow. No rat showed severe impairment.
Mechanical stimulation-induced pain of bilateral hind limbs

Mechanical pain thresholds of bilateral hind limbs were 13±3.16 g (left) and 14±6.21 g (right) at baseline in all rats. At day 1 after the BP AI, the mechanical pain thresholds of bilateral hind limbs significantly decreased to 5.67±0.82 g (left) and 5.67±0.82 g (right) in all the six groups comparing with baseline data (P<0.05). The threshold in 10 individuals even decreased to 2–4 g. The mechanical pain threshold in all rats increased to 6±1.26 g (left) and 7±1.67 g (right) after 6 days. On day 28, the mechanical pain threshold of bilateral hind limbs was 7±1.10 g (left) and 7±1.10 g (right), which was significantly lower than the baseline value (P<0.05). The threshold in the control group was unchanged during the 28 days. The mechanical pain thresholds of bilateral hind limbs during the 28 days are shown in Figure 2A.

Thermal stimulation-induced pain of bilateral hind limbs

BEAM value was set at 5, and the withdrawal time was 9.75±1.37 seconds (left) and 9.71±3.11 seconds (right) at baseline in all rats. Compared with the change of mechanical pain threshold, thermal pain threshold slightly decreased to 8.85±1.3 seconds (left) and 8.39±1.80 seconds (right) at day 1 after BP AI and recovered to 8.84±1.68 seconds (left) and 9.64±2.38 seconds (right) at 2 weeks after the procedure. The threshold of control group was unchanged during the 28 days. No statistical significance was found when comparing with the baseline data (P>0.05). The thermal thresholds of bilateral hind limbs at each time point are shown in Figure 2B.

Cytological study of GFAP

The expression of GFAP was elevated in the spinal cord of the paralyzed side on post-BP AI on day 1 (mean, 23/mm²; P<0.05) or the intact side (mean, 23/mm²; P<0.05). The GFAP expressions at each time point are shown in Figure 3.

Immunohistochemistry study of Iba-1

In BP AI rats, the expression of Iba-1 in the bilateral spinal cord on day 1 post-BP AI was 50/mm² (averaged; left) and 41/mm² (right). Compared with the control group (25/mm² on the left side and 26/mm² on the right side), BP AI-induced Iba-1 expression significantly increased and continuously increased on both sides on day 4 (50/mm², 32/mm²), 7 (41/mm², 37/mm²), 14 (47/mm², 41/mm²), 21 (41/mm², 35/mm²), and 28 (40/mm², 28/mm²). The expression of Iba-1 significantly increased on both sides in the BP AI rats when compared with the control group (P<0.05). On day 1, 4, and 28, when compared with the intact side (41/mm², 32/mm² and 28/mm², respectively), the expression of Iba-1 significantly increased on the paralyzed side (50/mm², 50/mm² and 40/mm², respectively; P<0.05). The Iba-1 expression at each time point is shown in Figure 4.

Discussion and conclusion

This study introduced a modified model of neuropathic pain by BP AI in adult rats. Immunohistochemistry study on GFAP (astrocyte) and Iba-1 (microglia) of the spinal cord sections and behavioral observations were performed. The outcomes demonstrated that our rat model can simulate BP AI and may be used to investigate the mechanism of neuropathic pain.

Discussion

The modified rat model

The classified neuropathic pain model was proposed by Gary and Bennett in 1998.12 They reported that chronic constriction...
Neuropathic pain rat model in brachial plexus avulsion injury

Based on our clinical experience, we found that a great number of BPAI patients were suffering from neuropathic pain. Therefore, we established BPAI model in adult rats to verify its feasibility in imitating the symptoms and course of neuropathic pain. Previous researches tended to use the anterior cervical–thoracic approach to establish BPAI model. In clinical practice, branchial plexus roots are avulsed in the vertebral canal. However, it is difficult to determine whether the avulsion site of nerve root is pre- or post-ganglionic.

Figure 3 Immunohistochemistry for GFAP in bilateral spinal dorsal horns in normal rats and BPAI rats.

Notes: (A) GFAP expression of the normal group. (B–G) GFAP expression of the experimental group at 1, 4, 7, 14, 21, and 28 days after BPAI. (H) Comparison of changes in the GFAP expression at 0, 1, 4, 7, 14, 21, and 28 days between the two groups. *Significant differences between the two groups, P<0.05; #significant differences between the two sides in the same group, P<0.05.

Abbreviations: BPAI, brachial plexus avulsion injury; GFAP, glial fibrillary acidic protein; L, left side; R, right side.

injury (CCI) rat model, which showed symptoms similar to that of human neuropathic pain, mainly manifested spontaneous pain and hyperalgesia on thermal and mechanical stimulation. Except for the CCI, partial sciatic nerve ligation (PSNL), spinal nerve ligation (SNL), spared nerve injury (SNI), and rhizotomy models have also been used for experimental investigations on neuropathic pain.9,13–15 However, these models simply imitated the symptoms of neuropathic pain of human, but not the underlying pathogenesis.
Except that, the lower brachial plexus nerves were difficult to expose, and the subclavian vein might be injured. In our study, we used posterior cervical–thoracic approach to establish the rat model. First, we could avulse the nerve root under direct vision at pre-ganglionic lesion. Second, the success rate and accuracy rate of the posterior cervical–thoracic approach is higher. In our experiment, we observed that all rats in the BPAl groups showed complete paralysis in the left forelimbs. No significant instability of the cervical vertebra was observed after the hemilaminectomy of C5–T1.

BPAl-induced neuropathic pain
In previous studies, the indexes for assessing neuropathic pain in animals were spontaneous pain and alldynia. The behavior of spontaneous pain in the paralyzed limb was hard to assess quantitatively in rats. The observations of allodynia were mainly the changed responses of the paralyzed limb with mechanical, thermal, and cold stimulations. However, BPAl rats lost the motor and sensory functions in the paralyzed forelimbs, and previous methods were no longer applicable. Rodrigues-Filho et al found that the pain threshold of bilat-
eral hind limbs decreased after BPAI in rats. Severe peripheral
nerve injury leads to neuropathic pain of injured limb and
subsequently induced pain hypersensitivity in intact limbs.
Therefore, mechanical and thermal pain tests in the intact
hind limb were applied for behavioral observation instead
of the injured forelimb.

Previous studies have proposed the possible mechanisms,
and central sensitization plays an important role in the
initiation and progression of neuropathic pain after BPAI.
Central sensitization is the enhancement in the function of
neurons and circuits in nociceptive pathways, which caused
by increase in membrane excitability and synaptic efficacy
or reduced inhibition. It is a manifestation of the remarkable
plasticity of the somatosensory nervous system in response
to activity, inflammation, and neural injury. Central
sensitization is characterized by the long-term potentiation
(LTP) of neuron. After peripheral nerve injury, damaged
and non-damaged A- and C-fibers begin to generate spontaneous
action potentials, which is a form of ectopic input. Such input
in A- and C-fibers begins to drive central sensitization in the
dorsal horn of spinal cord. Then injured and non-injured
sensory neurons in dorsal root ganglion change their mem-
brane properties and transmitter function. Factors such as
substance P, brain-derived neurotrophic factor (BDNF), and
the synthetic enzymes for tetrahydrobiopterin, an essential
cofactor for NOS, are released and drive central sensitiza-
tion. The previous study also found that A-ß fibers sprout
into laminae I–II of the dorsal horn and make contact with
nociceptive-specific neurons. Then nociceptive-specific
neuron, which receives noxious stimuli normally, begins to
receive input from non-noxious tactile stimuli and generate
pain. The previous study also found that spinal glial cells,
such as astrocyte and microglia, contribute to the induction
of C-fiber LTP via NMDA receptor. The reduction of the
synthesis, release, and activity of inhibitory transmitters also
contribute to central sensitization. The death of inhibitory
interneurons in the dorsal horn of spinal cord partly con-
tributes to the loss of GABAergic and decreased glycineric
inhibitory current.

Cortical plasticity may also take part in the process of
neuropathic pain. After peripheral nerve injury, the brain
representation maps in cortical and subcortical areas rear-
range. Falconer found that rhizotomy could not improve
the phantom limb pain of BPAI patient, while cordotomy
and dorsal root entry zone procedure could improve it.
However, functional magnetic resonance image (fMRI),
transcranial magnetic stimulation (TMS), and electrophysiological study still need to further investigate the
relationship between cortical plasticity and neuropathic
pain after BPAI.

Teixeira et al proposed that BPAI contributes to a direct
injury of the spinal cord. On the same side and contralateral
to the lesion, the loss of neuron in the dorsal horn was observed
at various levels. Neuronal death may contribute to this
phenomenon via the release of cytokines and chemokines,
glial cells activation, and neuronal apoptosis.

In our modified BPAI model, the changes in pain thresh-
old of bilateral hind limbs was observed and assessed. These
finding are consistent with previous studies.

Activation of astrocyte and microglia in neuropathic
pain
Glia, which makes up 70% of all cell types in CNS, is
an essential part of CNS. Astrocyte, oligodendrogia, and
microglia are the three kinds of glial cells. Early researches
considered that the occurrence and modulation of pain are
associated with neurons; glial cells are closely involved in
the process of pain. Since the early 1990s, scientists found
that glial cells are also involved in some information com-
munication processes such as signal transduction, receptor
expression, and transmitters expression. It was also reported
that glial cells, especially astrocyte and microglia, play a
crucial role in neuropathic pain. Studies demonstrated that
microglia and astrocytes in the spinal cord are activated
to express pro-inflammatory cytokines, chemokines, and
nerve active substance, which resulted in hyperexcitability
of neurons in the spinal dorsal horn.

Microglia are activated at the early stage after BPAI and
appeared to play an important role in the formation of the pain.
In neuropathic pain status, microglia are rapidly evoked and
the morphology, proliferative potential, gene expression, and
function of microglia are changed. Microglia express several
receptors for neurotransmitters, including glutamate recep-
tors, GABA receptors, cholinergic receptors, adrenergic recep-
tors, dopamine receptors, and purinoreceptors. Other receptors
include the receptors for histamine, opioids, cannabinoids,
substance P, neurotrophins, chemokines, interleukins (ILs),
interferons, tumor necrosis factor (TNF-α), and pathogen-
associated microbial patterns. Activation of the microglial
receptors leads to cellular signal transduction through
many essential intracellular pathways such as MAP kinases
(MAPKs), nuclear factor (NF)-kB, JAK/STATs, and PI3K/
AKt. Recent studies have revealed that inhibition of some
important microglial pathways is an effective way to dimin-
ish neuropathic pain states. Paszczuk et al used cannabinoid
agonists to inhibit NF-xB pathway to relieve neuropathic pain.
Astrocytes were activated by cytokines released from microglia and neurons. It seemed that astrocytes were more closely associated with the maintenance of neuropathic pain than microglia. In neuropathic pain status, the MAPK in astrocyte is activated by the pro-inflammatory cytokines, TNF-α, and produces CCL2, which is critical for the development and maintenance of neuropathic pain.\textsuperscript{30,31} Lu et al\textsuperscript{32} demonstrated that TNF receptor-associated factor 6 (TRAF6) upregulated in spinal cord astrocytes in the late phase after nerve injury, which maintained neuropathic pain by integrating TNF-α and IL-1β signaling and activating the JNK/CCL2 pathway in astrocytes.

In our study, the activation of glial cell was consistent with previous studies. However, in our study, the mechanism by which astrocytes were activated in the affected side, while microglia were activated in the bilateral spinal cord still need further investigation. Therefore, we can investigate the mechanism and treatment of neuropathic pain followed BPAI by using this model.

Conclusion
In the present study, we successfully established a modified model of neuropathic pain in rats by producing BPAI. Astrocyte and microglia were obviously involved in the formation and progress of neuropathic pain. Further investigations on the glial cells may provide a potentially rewarding method for treating neuropathic pain.

Abbreviations
BPAI, brachial plexus avulsion injury; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; CCI, chronic constriction injury; PSNL, partial sciatric nerve ligation; SNL, spinal nerve ligation; SNI, spared nerve injury; CNS, central nervous system.

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Author contributions
All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
There has been no significant financial support for this work that could have influenced its outcome. The authors report no other conflicts of interest in this work.

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