

Original Research Article

Effect of rPMS on N-type calcium channel in rats with neuropathic pain

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Abstract

Purpose: To investigate the effect of repetitive peripheral magnetic stimulation (rPMS) on N-type calcium channel of rats with neuropathic pain (NP).

Methods: Thirty-two Sprague-Dawley (SD) rats were randomized into control, mock surgical, model, and rPMS groups. For the model and rPMS groups, rat NP models were made based on chronic constriction injury (CCI) model from January 2018 to June 2019; the mock surgical group was treated to expose the sciatic nerve, while the control group received no treatment.

Results: Compared to the control group, the model group demonstrated a prominent increase in spontaneous pain-like behaviors, threshold of claw withdrawal in reaction to mechanical stimulation, substance P, glutamic acid, calcitonin gene-related peptide (CGRP), and calcium current, with a decrease in paw withdrawal thermal latency (PwTL) ($p < 0.05$). In comparison to the model group, alleviated spontaneous pain-like behaviors, reduced threshold of claw withdrawal in reaction to mechanical stimulation, substance P, glutamic acid, CGRP, and calcium current rPMS, with increased PwTL were observed in the rPMS group ($p < 0.05$).

Conclusion: rPMS alleviates NP syndromes and inhibits the activity of N-type calcium channel in rats. This finding provides a theoretical basis and reference for the clinical application of rPMS in the treatment of NP.

Keywords: Repetitive peripheral magnetic stimulation (rPMS), Neuropathic pain, N-type calcium channel, Paw withdrawal thermal latency

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INTRODUCTION

Neuropathic pain (NP) is pain caused by pathological changes in the somatosensory nervous system, and may be associated with common diseases such as postherpetic neuralgia, trigeminal neuralgia, painful radiculopathy, diabetic neuropathy, HIV infection, leprosis, amputation, peripheral nerve injury, and

apoplexia [1]. Although the global prevalence of NP is 1.5 – 8 % [2], effective treatments are absent owing to limited knowledge about the molecular mechanism of NP in the study circle [3]. Repetitive peripheral magnetic stimulation (rPMS) produces stimulation on the muscles, nerves, or spinal cord root [4] in a safe and minimally invasive way [5]. Furthermore, a study by Lim *et al* [6] showed that rPMS effectively

reduced acute back pain, while Bourinet *et al* [7] reported that it increased the motor-evoked potential (MEP) of the swallowing muscles as an effective treatment of dysphagia.

Neuropathy frequently results in changes in the sodium, calcium, and potassium channels in the relevant nerves, and it affects the transmission of sensory signals between the spinal cord and the brain [7]. As a component in the formation of action potential and triggering of downstream physiological processes, a voltage-dependent Ca²⁺ channel (VDCC) may be HV-dependent, with N-type calcium channel (Cav2.2) [8], or LV-dependent based on the voltage dependence of calcium channel activation [9]. Cav2.2 exists in large amounts in the cell bodies and the tips of the dorsal root ganglion (DRG) [10], and it involves the release of mediated pain-like neurotransmitters, including substance P (SP), glutamic acid (Glu), and Calcitonin gene related peptide (CGRP) [11]. It has been found that 90 % of the calcium current in sympathetic neurons belongs to N-type calcium channel current [12].

In this study, a NP model was built for rats based on the chronic constriction injury (CCI) model to determine the effect as a reference for clinical application.

EXPERIMENTAL

Animals

A total of 32 male Sprague-Dawley (SD) rats weighing 245 – 315 g were purchased from the Hunan Slac Jingda Laboratory Animal Co., Ltd from January 2018 to June 2019, and equally and randomly divided into the control group (n = 8), model group (n = 8), mock surgical group (n = 8), and rPMS group (n = 8) after health screening and observation for any obvious abnormal behaviors. All the animal experiments were carried out at Animal Center of Ganzhou City People's Hospital (Ganzhou, China). Every procedure was approved by the Animal Care and Use Committee of Ganzhou City People's Hospital (approval no. PJ2020-0256). All procedures involving laboratory animals were performed according to the Guiding Principles for Research Involving Animals and Human Beings [13].

Main instruments and reagents

SP and CGRP test kits were purchased from ADL, the United States; MagPro R30 magnetic stimulator from Mag Venture (Farum, Denmark); patch-clamp amplifier from AXON (New York, NY, USA); and glass microelectrode controller

from Narishige (Tokyo, Japan). Glass microelectrodes were purchased from Nanjing Quanshui Teaching and Experimental Equipment Co. Ltd (Nanjing, China); HP-1100 HPLC from Agilent (Santa Clara, CA, USA); deoxyribonuclease; from Sigma (St. Louis, MO, USA); while Hank's solution, protease, and collagenase were obtained from Gibco (Rockville, MD, USA).

RMT acquisition

Resting motor threshold (RMT) is defined as the minimum stimulus intensity that produces a suprathreshold reaction by triggering stimulation at $\geq 50\%$ at the best stimulating spot when the person or animal to be tested is at rest.

Seven healthy rats were anesthetized (with 10 % chloral hydrate injected into the abdominal cavity at a dose of 300 mg/kg (body weight), and stimulated using a magnetic stimulator which surface electrode was placed at the left gastrocnemius. The reference electrode was at the ankle joint, and the grounding wire was connected to the rat's tail. By moving the coils, the optimal stimulating spot was identified, and the stimulation intensity was gradually raised from the value at which no MEP response was incurred, and applied 10 times in each intensity grade at an interval of 7 sec, until an MEP response was observed in all 10 attempts. The mean stimulation intensity at this point was recorded for the seven rats as mean RMT.

NP modeling

For the model group and the rPMS group, rat NP models were created based on the CCI model. Anesthetized rats were secured on the operating table in a dorsal position to shave off the hair on the thighs, in order to expose the skin that was then disinfected and cut through at the side. Using a knife handle, the musculus biceps and the musculus vastus lateralis were separated to expose the sciatic nerve and its branches. Four mild ligature rings made of 4-0 chromicized catgut were placed around the trunk of the sciatic nerve at every 1 mm while avoiding the blood vessels, until the calf muscles trembled lightly. The incision was sutured and disinfected. For the mock surgical group, the sciatic nerve was exposed. However, the incision was left untreated. The control group received no treatment.

rPMS treatment

The rPMS treatment was given to rats in the rPMS group on postoperative day 3, with the

primary motor cortex of the right brain (M1) as the stimulating spot. The magnetic stimulating coil was placed parallel to the projection area of the skull corresponding to M1 on the body surface, and the stimulation intensity was set to 90 % RMT with 1,200 pulses at the frequency of 20 Hz for 3-s stimulation before a break of 27 sec. With 20 stimulations in 10 min, the rPMS treatment was administered once daily for 10 d.

Assessment of pain behavior

The experimental rats were assessed for spontaneous pain-like behavior and measured for threshold of claw withdrawal in reaction to mechanical stimulation and paw withdrawal thermal latency (PwTL) at T₀ (day 0), T₁ (day 1), T₂ (day 1), and T₃ (day 3). Assessment of spontaneous pain-like behavior included excessive paw licking, toe tightening, reduced bearing capacity of the injured limb, limping, and autophagia according to Kawakami method [14], in which, 0 indicates normal motor behavior without posterior claw deformity, 1 indicates normal motor behavior with obvious posterior claw deformity, 2 suggests mildly abnormal motor behavior with drop foot, and 3 reflects severely abnormal motor behavior with paralytic posterior claw at the surgical side. The score for each group was calculated and expressed as mean ± standard error of mean (SEM) values.

Assessment of PwTL

The heat tolerance of the pelma of injured limb was measured 3 times every 5 min with a thermalgesia tester. The period from exposure to the lifting of the posterior claw was referred to as PwTL.

Measurement of the threshold of claw withdrawal

A dynamic acupuncture pain threshold detector was used to measure the minimal pressure resulting in the withdrawal reaction of the rats for 3 times at a frequency of once every 5 min, in order to obtain the threshold of claw withdrawal in reaction to mechanical stimulation.

Calcium current test

After the last pain behavior assessment at T₃, four rats from each group were randomly selected, euthanized and placed in 0 °C Hank's solution for rapid removal of the glomeruli of the sympathetic ganglia. Thereafter, the glomeruli of the sympathetic ganglia was put in 4 °C oxygen-saturated medium. The connective tissues were cleared away, chopped, and digested at 31.5 °C

in a medium containing 1.5 mg/mL protease, 1.0 mg/mL collagenase, and 0.1 mg/mL deoxyribonuclease via oscillation, and euthanized so as to harvest the free sympathetic ganglion cells into the perfusate that was stored at room temperature for a maximum time of 5 h.

The calcium current of the sympathetic neurocytes was recorded for each group using the whole-cell patch-clamp technique at a clamping voltage of -80 mV and a stimulation voltage of 10 mV for 400 ms. For each group, the results of the 10 cells were recorded and averaged to obtain the calcium current.

Assessment of SP, Glu, and calcitonin gene-related peptide (CGRP) release levels

After the last pain behavior assessment at T₃, 4 rats were randomly selected from each group and beheaded by breaking their necks. 2 mL blood via cardiac puncture was sampled, collected in a test tube containing 10 % EDTA (30 µL)-trasyolol (40 µL) and centrifuged for 10 min at 3 × 10³ r/min at 4 °C. The liquid supernatant was recycled and stored at -80 °C in a refrigerator for future test.

The content of Glu was determined using HPLC; substance P and CGPR content were measured using ELISA in strict accordance with the instructions.

Statistical analysis

The data were entered into Statistical Package for Social Sciences (SPSS) 20.0 (IBM, Armonk, NY, USA) for statistical analysis. Measurement data were expressed as mean ± SEM, while other data was presented as number of cases (n) and percentages (%). One-way analysis of variance and Tukey's HSD test were applied to determine statistical difference, and $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Pain behaviors

Figure 1 shows the proportion of the scores of each group for spontaneous pain-like behaviors at various stages. There was no statistical difference in the scores for spontaneous pain-like behaviors between the mock surgical group and the control group in the same stage ($p > 0.05$). At T₁, T₂, and T₃, the scores for spontaneous pain-like behaviors in the model group had statistical difference compared to the control group ($p < 0.001$). At T₁, there was no statistical difference

in the scores for spontaneous pain-like behaviors between the rPMS group and the model group ($p > 0.05$), while at T₂ and T₃, the two groups had statistical differences in the scores (T₂: $p = 0.042$ and T₃: $p = 0.002$). In the rPMS group, the score for spontaneous pain-like behaviors showed statistical differences between T₂ and T₁ ($p = 0.041$).

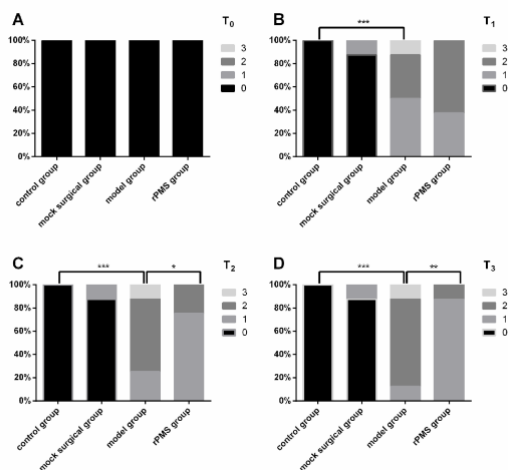


Figure 1: The Proportion of the score of each group for spontaneous pain-like behaviors in the various stages. (A: T₀; B: T₁; C: T₂; D: T₃; * $p < 0.05$, *** $p < 0.001$)

Rats in the model group and the rPMS group showed obvious claw withdrawal at T₁ and decreased threshold of claw withdrawal in reaction to mechanical stimulation ($p < 0.001$). At T₁, the model group and rPMS group were different ($p < 0.001$) from the control group in terms of decrease in threshold. From T₁ to T₂,

and from T₂ to T₃, the rPMS group exhibited a rise in the threshold of claw withdrawal in reaction to mechanical stimulation ($p < 0.01$), outnumbering the model group at the same stage ($p < 0.01$, Table 1).

Although no statistical difference was observed among the experimental rats in terms of PwTL at T₀ ($p > 0.05$), rats in the model group and the rPMS group showed obvious heat reaction at T₁ and decrease of PwTL ($p < 0.001$). At T₁, the control group and the mock surgical group failed to achieve statistical difference ($p > 0.05$) in PwTL compared to that at T₀, while the model group and rPMS group were statistically different ($p < 0.001$) from the control group in terms of PwTL decrease. From T₁ to T₂, and from T₂ to T₃, the rPMS group exhibited a rise of PwTL ($p < 0.01$), outnumbering the model group in the same stage ($p < 0.01$, Table 2).

Effect of rPMS on the current of N-type calcium channel

Figure 2 shows the increases in the calcium current in the mock surgical group, model group, and rPMS group when compared to that in the control group, meaning that the mock surgical group was not different from the control group ($p > 0.05$). However, compared to the control group, the calcium current in the mock surgical group and the rPMS group rose by $67.31 \pm 4.63\%$ ($p < 0.001$) and $43.08 \pm 2.48\%$ ($p < 0.001$) respectively, while a comparison between rPMS group and the model group revealed a decrease of $14.42\% \pm 3.06\%$ ($p < 0.05$).

Table 1: Threshold of Claw Withdrawal in Reaction to Mechanical Stimulation (g, n = 8, mean \pm SD)

Group	T ₀	T ₁	T ₂	T ₃
Control	38.14 \pm 3.46	36.13 \pm 4.61	35.64 \pm 3.99	34.16 \pm 3.67
Mock surgical	37.01 \pm 4.17	35.90 \pm 4.93	34.34 \pm 4.78	33.95 \pm 4.10
Model	37.89 \pm 3.70	14.14 \pm 1.36 ^{***###}	17.18 \pm 2.80 ^{###}	18.15 \pm 3.53 ^{###}
rPMS	38.04 \pm 3.52	15.78 \pm 2.58 ^{***}	22.04 \pm 4.11 ^{**▲▲}	25.94 \pm 2.39 ^{**▲▲}

(*** $p < 0.001$ and ** $p < 0.01$ in the same group when compared with the previous stage; ### $p < 0.001$ when compared with the control group in the same stage; ▲ $p < 0.01$ as compared with the model group in the same stage)

Table 2: PwTL (s, n = 8, mean \pm SD)

Group	T ₀	T ₁	T ₂	T ₃
Control	17.86 \pm 2.08	16.71 \pm 2.35	18.02 \pm 1.98	17.31 \pm 1.88
Mock surgical	17.28 \pm 1.89	16.88 \pm 3.42	17.66 \pm 2.83	17.15 \pm 2.33
Model	17.63 \pm 2.36	11.13 \pm 1.88 ^{***###}	11.94 \pm 2.07 ^{###}	12.31 \pm 2.43 ^{###}
rPMS	17.93 \pm 3.52	10.95 \pm 1.90 ^{***###}	13.63 \pm 2.67 ^{**▲▲}	16.00 \pm 2.17 ^{**▲▲}

*** $P < 0.001$ in the same group when compared with the previous stage; ### $p < 0.001$ compared with the control group in the same stage; ▲ $p < 0.01$ compared with the model group in the same stage

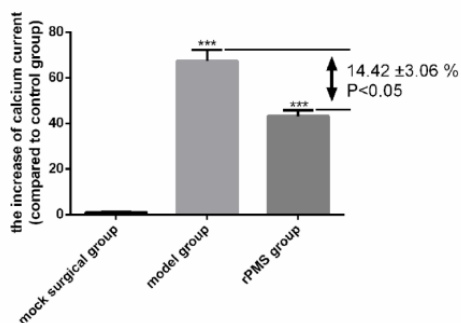


Figure 2: Increase in the calcium current in the mock surgical group, model group, and rPMS group. *** $P < 0.001$ when compared with the control group

Release level of neurotransmitters

As shown in Figure 3, compared to the control group, in terms of the release level of Glu, SP, and CGRP, the mock surgical group demonstrated no statistical difference, and the model group exhibited a rise ($p < 0.001$). Comparison between the rPMS group and the model group revealed a decrease in the Glu, SP, and CGRP levels ($p < 0.001$).

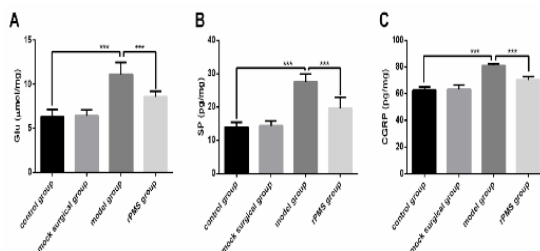


Figure 3: Release level of neurotransmitters in each group. A: level of Glu; B: level of SP; C: level of CGRP; *** $p < 0.001$

DISCUSSION

VDCCs play an important role in the release of neurotransmitters, in addition to its mediation of calcium ion transport in the neuronal cells. As a member of VDCCs, the Cav2.2 functions as the major calcium channel [15] for the release of neurotransmitters at the synapses of most mammals, and is closely associated with pain and related reactions. Owing to its induction, excitability rises and neurotransmitters are released in NP [8].

Intracellular calcium ions may participate in the development of persistent pain and increase the expression of VDCCs and ligand-dependent calcium channels in the neurons [16]. Hatakeyama *et al* [16] found that the removal of the Cav2.2 gene resulted in the almost

disappearance of the Cav2.2 current; however, it reduced nocuous reactions. In contrast, Leo *et al* [17] demonstrated the role of up-regulating the Cav2.2 channel in mediating CDDP-induced NP. Yang *et al* [10] showed that with peripheral nerve injury, the Cav2.2 in intact DRG was up-regulated under the induction of IL-1 β , thus leading to an increase in the neuronal excitability of the injured nerves and resulting in NP. In this study, the NP model rats were treated with rPMS to study its effect on calcium current and the release of related neurotransmitters in the Cav2.2 to assess the efficacy of rPMS on NP.

Based on the assessment of the pain behaviors in this study, the rPMS group was alleviated in terms of spontaneous pain-like behaviors, and reinforced in resistance against mechanical stimulation and heat ($p < 0.05$), compared to the model group after rPMS treatment. Momosaki *et al* [18] believed that rPMS may improve the patients' sensory functions, while Flamand *et al* [19] discovered the regulating role of rPMS in peak-peak amplitude of MEPs. Krause *et al* [20] revealed the function of rPMS in regulating motor cortex excitability. Changes in these features or functions may result in the recovery of some somatosensory functions of the NP model rats, thereby relieving pain.

Changes in N-type calcium channel current of the sympathetic neurons indicated that rPMS may weaken the current of the N-type calcium channel, while its role of inhibiting the release of SP, Glu, and CGRP was supported by the test results of the neurotransmitter levels. Perret *et al* [21] showed that the Cav2.2 channel expressed itself at the front tips of the synapses to open subunits as a way of facilitating the release of SP, Glu, and CGRP. The discovery of the weakened current in the N-type calcium channel in the rPMS group rats indicate that the activity of the Cav2.2 may be reduced, resulting in less opening subunits, and the inhibited release of SP, Glu, and CGRP, because it may cause NP [22]. The inhibitory effect of rPMS on those neurotransmitters may be the cause of catabatic pain syndrome in rats because SP may be connected with a series of senses by binding with NK1 [23]. The release of Glu may induce the activation of C-fiber, leading to pain [24]; and CGRP induced the synaptic pain signal pathway via second messengers, including protein kinases A and C.

This study assessed the effect of rPMS on the current and release level of neurotransmitters in the Cav 2.2 in a rat NP model. The mechanisms underlying the Cav2.2 are not limited to the two, according to Yang *et al* [10], who demonstrated

the involvement of IL-10 and IL-1 β with Cav2.2 and pain. Therefore, future studies should investigate the effect of rPMS on IL-10 and IL-1 β , or the change in the associated proteins in Cav2.2 to further understand the relationship between rPMS and Cav2.2.

CONCLUSION

The rPMS weakens calcium current and inhibits the release of neurotransmitters by depressing Cav2.2, thereby providing relief from NP. Therefore, rPMS can potentially be developed for the management of NP.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xianbin Cai and Xuefen Xu designed the study and performed the experiments, Xianbin Cai collected the data, Xuefen Xu analyzed the data, Xianbin Cai and Xuefen Xu prepared the manuscript. All authors read and approved the final manuscript.

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