Original Paper

Cerebellar Fastigial Nucleus Electrical Stimulation Alleviates Depressive-Like Behaviors in Post-Stroke Depression Rat Model and Potential Mechanisms

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Key Words

Cerebellar FNS • PSD • rCBF • Cerebellar Purkinje cells • Cytokines

Abstract

Objective: To identify the molecular mechanism of post-stroke depression (PSD), and observe the therapeutic effects of cerebellar fastigial nucleus electrical stimulation (FNS) on the behaviors and regional cerebral blood flow (rCBF) in a PSD rat model. **Methods:** Healthy SD rats were randomly divided into four groups (sham, stroke, post-stroke depress and FNS group). Sham group (n = 6) underwent sham operation. The other three groups (n = 6*3) underwent MCAO. Rats were examined twice a week in open field test. Moreover, neuroprotective effect on cerebellar Purkinje cells and expression of cytokines in hippocampal tissue were examined. **Results:** The PSD group showed a significant weight loss, decreased consumption of sucrose water, reduced rearing and locomotor activities. The FNS significantly alleviates the body weight loss and sucrose preference, locomotor and rearing activities. The bilateral rCBF was also restored after FNS treatment. Moreover, FNS improved the neuroprotection via suppressing apoptosis of cerebellar Purkinje cells. And the inflammatory cytokines mRNA level in hippocampus was significantly decreased. **Conclusion:** FNS treatment alleviates depressive-like behaviors and rCBF in PSD rats model, which could be attributed to its ability to protect cerebellar Purkinje cells and decrease the mRNA level of inflammatory cytokines.

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Introduction

One of the most frequent psychiatric complications of stroke is post-stroke depression (PSD). Its incidence is about 33–40% in stroke patients and approximately 3 million Americans are affected each year [1, 2]. Stroke survivors may have difficulty in identifying

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and explaining symptoms of depression, especially older stroke survivors with higher morbidities. Stroke patients with PSD are at higher risk of death and achieve a mild improvement in rehabilitation programs compared with stroke patients without depression. As a special psychiatric disorder and most common complication following stroke, PSD is usually tricky to treat [2].

Gliogenesis and neurogenesis are formed as an integral part of the adaptive reaction of the brain responding to ischemia. Likely, it has been reported that hippocampal neurogenesis is enhanced by transient global ischemia in rats [3, 4]. Ischemia adequately enhances proliferation of resident glial cells in majority of non-neurogenic regions of the adult brain [5, 6]. In a recent study, unexpected chronic minor stress following left MCAO has been shown to cause depressive-like behaviors as well as reduced degrees of hippocampal neurogenesis in rats [7, 8]. Moreover, anti-depressant therapy with citalopram reversed both the antineurogenic and the behavioral impacts of persistent stress following brain ischemia [8]. Recently, it was reported that the cerebellum may exhibit a role in psychiatric illness, behavior and cognition [9, 10]. Cerebellum dysfunction might be involved in pathogenesis of PSD, based on the correlation between the cerebellum and the affective disorder, similarity in pathology between PSD and neuropsychiatric diseases (particularly for depression), and the clinical trial indicating that cerebella substantially relieves symptom of PSD upon activation [10]. But there is little evidence that investigates the putative cerebellar effect in PSD [11].

Several clinical studies suggested that symptom of PSD can be substantially eased by activated cerebella. Sui et al. discovered that emotion disorders in post-stroke patient can be improved by fastigial nucleus electrical stimulations [12]. Likely, Su et al. reported that electrical stimulation cerebellar fastigial nucleus showed substantially alleviated symptom of patients who had PSD, in comparison to healthy controls [13]. Electrical stimulation excites fibers passing through the fastigial nucleus, leading to increased blood pressure, reflexive vascular expansion, and increased rCBF [14]. FNS can pass fibers exceeds the sympathetic inhibitory response by FN nerve cells. FNS can significantly enhance the tolerance of brain tissue to subsequent cerebral ischemia. Robust data show that electrical stimulation of the cerebellar fastigial nucleus can elicit marked global protection against brain injury in rats [15-17]. Actually FNS as a therapeutic choice is available in our hospital to treat some mental illnesses including PSD.

Many studies have sought to explore the mechanisms that might account for the neuroprotection elicited by FNS. We focus on those mechanisms and the clinical applications of FNS in treatment of PSD. Postmortem brains of patients with depression exhibit neuronal apoptosis and DNA fragmentation, indicating an increased neuronal vulnerability in patients with depression [18]. In animal models of mood disorders, reduced neurogenesis and neuronal loss have also been found [18-20]. Two mechanisms may involve in neuronal cell death, the delayed form, apoptosis, or an acute form, or necrosis that happens rapidly [21].

In this study, we showed that the FNS treatment significantly alleviated the body weight loss and depressive-like behaviors. Further studies showed that the rCBF were significantly increased by the FNS, compared with the PSD rats. The neuroprotection of cerebellar Purkinje cells mediated by FNS were confirmed with electronic microscope and immunostaining of activated caspas-3.

Materials and Methods

Establishment of PSD rat model

The Sprague-Dawley rats were obtained from Animal Center, Liaoning Medical College and maintained in plastic cages. The conditions were controlled at room temperature and relative humidity of 50–60%. The rats were fed with tap water and standard rat diet. Twenty four healthy Sprague Dawley rats with uniform behavior were selected and randomly divided into four groups as follows: Sham, stroke, PSD and FNS groups. In the sham group, sham operated rats underwent the same surgical procedure, except the suture was not introduced into the artery. The rats underwent MCAO and divided into 3 groups: stroke,

PSD and FNS group. The PSD and FNS rats were subjected to isolation-housing in combination with chronic unexpected mild stress (CUMS, water deprivation, wet litter, fasting, behavioral restriction, tail clamping, electric shock to foot and ice-water swimming) to set up a depression model. The study protocol was approved by the research ethics committee at Liaoning Medical College.

Cerebellar fastigial nucleus electrical stimulation

Rats were fasted for 8–12 hours, 3.5% (w/v) chloral hydrate (10 mL/kg, intraperitoneally) was used to anesthetize the rats, and a brain stereotactic endoscope (Japan Mau Science Equipment Research Institute, Tokyo, Japan) was used to immobilize the rats in accordance with the manufacturer's protocol. Stereotaxic atlas of the rat brain were used to accurately position the fastigial nucleus, and set the posterior border of the anterior fontanelle as the zero point, an 11.1-mm incision performed, YC-2 programmed electrical stimulation instrument (Chengdu Instrument Factory, Chengdu, Sichuan Province, China) with a 70- μ A direct-current square-wave pulse (50 Hz) was used to perform the attachment of electrodes in a hole made in the skull. All the tests were performed three times. The Rat body weights, consumption of sucrose water, rearing activity, locomotor activity, rCBF of all the rats were collected during the research.

Locomotor activity

The general locomotor activity were performed on the OFT (open field test). Rats were placed in the front right corner of a clear acrylic box (16" $\times$ 16") for 20 min. A computer operated PAS Open Field system (San Diego Instruments, San Diego, CA) was used to quantify the locomotor activity as the total number of beam breaks in accordance with the manufacturer's instruction.

Regional cerebral blood flow (rCBF)

A 2 MHz pulsed transcranial Doppler ultrasound (TCD) system (Spencer Technologies, Seattle, WA, USA) was used to measure the rCBF in the middle (MCAv) (Contra) and posterior (PCAv) (Contra) cerebral arteries continuously throughout each experiment in accordance with the manufacturer's instruction. The standardized procedures were used to determine the location and identification of MCA (Contra) and PCA (Contra) as described by Willie et al. [22]. A headband fixation device (Mark600, Spencer Technologies, Seattle, WA, USA) was used to fix and hold the Bilateral TCD probes

Electron microscopy

Rats were euthanized and the sections on adjacent grids during photography. The sections were stained with uranyl acetate and lead citrate, and were analyzed using a Philips TEM/CM 10 electron microscope.

RNA isolation and real-time PCR

TRIzol reagent (Invitrogen, California, USA) was used to extract the total RNA from tissue samples according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) was used to evaluate the mRNA level of TNF- α , IL-6, and IL-1 β mRNAs levels. Superscript II Reverse Transcriptase (Invitrogen, CA, USA) was used to reverse transcribe the RNA isolated to cDNA with an oligo dT18 primer in accordance with the manufacturer's instruction. The Sequence Detection System 7900HT (Applied Biosystems, Foster City, CA) containing the Universal Master Mix (PE Applied Biosystems, Foster City, CA) was used to perform the real-time PCR with specific primers in order to quantify the mRNA levels. All tests were performed at least three times. The GAPDH was used as an internal control for normalization of TNF- α , IL-6, and IL-1 β mRNAs expression. The $\Delta\Delta$ Ct method was used to calculate the expression of the cytokines. All tests were performed at least three times.

Immunohistochemistry

Immunohistochemistry was performed on brain sections using a rabbit polyclonal antibody directed against Cleaved caspase-3 (cell signaling technology, #9664). Animals were perfused using 4% paraformaldehyde in PBS. Brains were post-fixed in the same fixative overnight at 4°C and cryoprotected overnight at 4°C in PBS containing 30% sucrose. Ten micrometer parasagittal sections were cut using a cryostat and incubated overnight at 4°C using Cleaved caspase-3 antibody (dilution 1:1000). Immunocomplexes were then revealed using HRP (horseradish peroxidase)-conjugated secondary antibody was used to treat the sections at room temperature for 30 min. 3, 3'-diaminobenzidine tetrahydrochloride solution was used to treat the sections, Hematoxylin and eosin (H&E) stains was used to counterstain all the sections.

Statistical analysis

All data shown here were in the form of mean $\pm$ SD (standard deviation) unless specially specified. SPSS15.0 software (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analysis, the comparisons between two groups were carried out using Student's t-test, and comparisons among three groups were done using ANOVA, occurrence analysis were performed using Chi-squared test. A p-value less than 0.05 were considered significant statistically.

Results

Establishment of the post-stroke depression rat model

Healthy Sprague Dawley rats were subjected to MACO. The PSD and FNS rats were further in combination CUMS to develop depress rats model. The FNS group were treated with FNS as described in the methods section. One week after the initiation of the treatment, more symptoms including slow response, drowsiness and sluggishness were recorded without any unexpected death.

Cerebellar FNS alleviates depressive-like behaviors

In our post-stroke depressive-like rat model, PSD group show depressive-like behaviors, such as body weight-loss (Fig. 1), sucrose preference (Fig. 2), locomotor activities (Fig. 3) and rearing activities (Fig. 4) in OFT. After FNS treatments, the rats significantly gain body weight (since day 21st) and sucrose preference (since day 17th), locomotor activities and rearing activities in open field test (since Day 21st). These data indicate that cerebellar FNS significantly alleviates depressive-like behaviors in the post stroke depressive-like rat models.

Fig. 1. Dynamic changes in rat body weight. Rat body weights in the stroke and FNS groups were reduced before Day 11, and subsequently the body weights increased. The body weights in the PSD group continually decreased and reached their lowest level on Day 30. $\nabla P < 0.01$, vs. stroke group; $\star P < 0.05$, vs. PSD group. The bars represent SD. N=6.

Fig. 2. Dynamic changes of sucrose preference. The consumption of sucrose water decreased in the PSD and the FNS groups during the first 11 days compared with sham-operated and stroke groups. Subsequently, the consumption in the FNS group increased slightly from Day 14 and remained constant thereafter. The consumption of sucrose water in the PSD group continually decreased and reached the lowest level on Day 25. $\nabla P < 0.01$, vs. stroke group; $\star P < 0.01$, vs. PSD group. The bars represent SD. N=6.

Fig. 3. Dynamic changes of locomotor activity in the open-field test. The scores for locomotor activity in the sham-operated and stroke the groups were not significantly different. Locomotor activity in the PSD group was significantly reduced compared with the stroke group and reached its lowest point on Day 30. Locomotor activity in the FNS group decreased up till Day 17 and then it increased slightly. The difference between the PSD and FNS groups was statistically significant after Day 21. ▼ $P < 0.01$, vs. stroke group; ★ $P < 0.01$, vs. PSD group. The bars represent SD. N=6.

Fig. 4. Dynamic changes of rearing activity in the open-field test. The scores for rearing activity in the sham-operated and stroke groups were not significantly different. Rearing activity in the PSD group was significantly reduced compared with the stroke group and reached its lowest point on Day 30. Rearing activity in the FNS group decreased up till Day 17 and then it increased slightly. The difference between the PSD and FNS groups was statistically significant after Day 21. ▼ $P < 0.01$, vs. stroke group; ★ $P < 0.01$, vs. PSD group. The bars represent SD.

Fig. 5. Dynamic changes of bilateral rCBF. The cerebellar rCBF (contra) in the stroke group was reduced on Day 1 ($P < 0.05$) compared with the sham-operated group, and then it increased and recovered after Day 17. The cerebellar rCBF (contra) in the PSD group significantly decreased compared with the stroke group until the lowest point was reached on Day 25. For the FNS group, cerebellar rCBF (contra) had a similar rCBF profile as that of the stroke group except it had a relatively higher rCBF. ■ $P < 0.05$, vs. sham-operated group; ▼ $P < 0.01$, vs. stroke group; ★ $P < 0.01$, vs. PSD group. The bars represent SD.

Cerebellar FNS significantly restore bilateral rCBF

The enhancement of rCBF is tightly and positively coupled to increases in synaptic glucose metabolism and electrical activity [23, 24]. We also examine if Cerebellar FNS affects the rCBF in the PSD rat model. our data show that the rats in PSD group was significantly lost their rCBF since day 7th; moreover, the FNS treatments could significantly increase the rCBF in since day 5th (Fig. 5).

Cerebellar FNS protects cerebellar Purkinje cells

Cerebellar Purkinje cells are well-known for synaptic plasticity. Here, we investigated the ultrastructure of the cerebellar Purkinje cell terminals in the cerebellar nuclei as well as the activities of their target neurons. In the sham and stroke groups ultrastructure of the Purkinje cells (contra) in the stroke rats appeared slightly damaged (Fig.6 B) comparing with Sham rats (Fig. 6A). The ultrastructure of Purkinje cells in PSD rats showed clear signs

Fig. 6. Neuroprotection of FNS on cerebellar Purkinje cells on Day 14. The ultrastructure of Purkinje cells (ipsi) in all groups remained mostly unchanged. The ultrastructure of the Purkinje cells (contra) in the stroke rats appeared slightly damaged (A, B). Magnification $\times 4000$ for A and $\times 14000$ for B. The ultrastructure of Purkinje cells in PSD rats showed clear signs of damage (C, D). Magnification $\times 3500$ for C and $\times 12000$ for D. Evidence of cellular damage was reduced in the FNS group (E, F). Magnification $\times 3500$ in E, and $\times 12000$ in F. ① = Expanded endoplasmic reticulum, ② = vague and swollen mitochondria. The images on the right are magnifications of the images on the left. Scale bars: $4\mu\text{m}$ in A, C, and E; $1\mu\text{m}$ in B, D, and F. N=3.

of damage (Fig. 6, C & D). Meanwhile, the cellular damage was reduced in the FNS group (Fig. 6, E & F).

In addition, we examined the pro-apoptosis marker, activated caspase-3, in the cerebellar Purkinje cells. Comparing with sham-operated rats (Fig. 7A), the immunostaining of activated caspase-3 is significantly higher in stroke group (Fig. 7B), and even higher in the PSD rats (Fig. 7C). The cerebellar FNS will dramatically decrease the immunostaining of caspase-3 (Fig. 7D), which indicated that the FNS protects the cerebellar Purkinje cells from apoptosis induced by PSD.

Cerebellar FNS decrease expression of inflammatory cytokines

Significantly higher levels of inflammatory markers are associated with a range of depressive symptoms. Here, we examine the mRNA level of the $\text{TNF-}\alpha$, IL-6, and IL-1 β in hippocampal tissue (Fig. 8, A-C, respectively). The stroke rats have a transient cytokines

Fig. 7. Effects of FNS on caspase-3 activation in cerebellar Purkinje cells. There were very few Purkinje cells that were immunostained for activated caspase-3 in the sham-operated group (A). The level of activated caspase-3 significantly increased ($P < 0.001$) in the stroke group (B) compared with the sham-operated group. Compared with stroke group, the level of activated caspase-3 in the PSD group (C) was significantly higher ($P < 0.001$). The level of activated caspase-3 in the FNS group (D) was significantly reduced ($P < 0.001$) compared with the PSD. $N=3$.

Fig. 8. Effects of FNS on expression of TNF- α , IL-6, and IL-1 β in hippocampal tissue. The expressions of TNF- α , IL-6, and IL-1 β mRNAs (A-C, respectively) in hippocampal tissue of PSD rats on Days 7, 14, and 21. Compared with the sham-operated group, the levels of cytokine mRNAs in the stroke group increased within 14 days, and then the levels decreased to their lowest levels on Day 21. Compared with the stroke group, the levels of the cytokine mRNAs in the PSD group significantly increased within 14 days, and then they remained mostly stable after Day 14. In the FNS group, mRNA levels displayed a similar profile as the PSD group, but the mRNA levels were significantly lower than the PSD group. ★ $P < 0.05$, vs. control group on Day 7; ☆ $P < 0.05$, vs. control group on Day 14; ∇ $P < 0.01$, vs. stroke group on Day 7; □ $P < 0.01$, vs. stroke group on Day 14; △ $P < 0.01$, vs. stroke group on Day 21; ▼ $P < 0.05$, vs. PSD group on Day 7; ■ $P < 0.05$, vs. PSD group on Day 14; ▲ $P < 0.05$, vs. PSD group on Day 21. The bars represent SD. $N=3$.

expression upregulation on day 7th and 14th, comparing with sham rats. The PSD rats have significantly upregulated expression of cytokine since day 7th to the day 21st. The PSD rats with FNS have significantly lower expression level of cytokines, which is consistency with the decrease of the depressive symptoms in the FNA rats.

Discussion

Post-stroke depression (PSD) is highly clinical relevance event. At least 1/3 of survivors from stroke present with mood symptoms sometimes following the event [1, 25, 26]. Depression following stroke often goes through a chronic phase and is correlated with diverse poor health outcome such as morbidity, mortality and aggravated disability [27-30]. Intriguingly, during the chronic phase following stroke, symptoms of depression may even aggravate [2]. Older patients with acute stroke experience depression after 20 months have poorer physical and cognitive outcome [31].

FNS renders the brain to prevent neuronal damage for up to 2 weeks following stimulation [15]. This inhibition of apoptosis provided by FNS was modest, being decreased by about 30% when compared to controls, and relatively lower than expected from *in vivo* studies in which there are more than 50% maximal decreases in cell death [15-17]. This may reveal the property of the *in vitro* system in that adult neurons are vulnerable in culture, so the complete protective effect of FNS is not fully reflected in this system. Moreover, *in vivo* neuroprotection by FNS may be associated with several protective mechanisms in addition to anti-apoptosis, including the systemic inhibition of inflammatory responses [32]. For instance, interleukins 1 and 6 (IL-1, IL-6) and tumor necrosis factor (TNF) had already been demonstrated to trigger short-term or acute hyperalgesia but were now involved directly in allodynia, chronic hyperalgesia and neuropathic pain [33, 34]. In this study, we found that the cerebellar FNS significantly alleviates the body weight loss (since day 21st) and sucrose preference (since day 17th), locomotor activities and rearing activities in open field test (since Day 21st). Moreover, the stroke rats have a transient cytokines expression upregulation on day 7th and 14th, comparing with sham rats. The PSD rats have significantly upregulated expression of cytokine since day 7th to the day 21st. The PSD rats with FNS have significantly lower expression level of cytokines, which is consistent with the decrease of the depressive symptoms in the FNA rats.

The region salvaged by FNS corresponds to neurons which are estimated to be experiencing a postponed death process, not those dying neurotically and immediately resulting from the insult. For instance, the tissue salvaged by FNS after a focal ischemic injury aligns with a zone of the focal ischemic infarction peripheral to the ischemic core, known as the presumed ischemic penumbra [35]. Neurons in the ischemic core exhibit irreversible injury over a short period from excessive anoxic stress [36]. By contrast, the penumbra is hypermetabolic and has a partially preserved blood supply, which causes sublethal damage finally facilitating postponed apoptotic cell death [37, 38]. Overall, these findings increase the intriguing potential that FNS may not only inhibit inflammatory reactions, but also alleviate apoptotic processes resulting in neuronal death after an ischemic event [32]. In this study, we conducted the immunostaining of activated caspase-3 and found that apoptosis is significantly higher in stroke group (Fig. 7B), and even higher in the PSD rats (Fig. 7C). The cerebellar FNS dramatically decreased the expression level of caspase-3 (Fig. 7D), suggesting that the FNS protects the cerebellar Purkinje cells from apoptosis induced by PSD.

Taken together, our study confirmed that Cerebellar FNS alleviates depressive-like behaviors in PSD rat model. Further mechanism study show that Cerebellar FNS protect Purkinje cells and decrease the expression level of cytokines in hippocampal tissue. In this study, we confirmed the dysfunction of cerebellar fastigial nucleus is involved in the pathogenesis of development of post-stroke depression; Secondly, poststroke depression experimental modelling could facilitate the development of novel therapeutic tool to treat post-stroke depression; Thirdly, such treatment may avoid the adverse effects caused by the medical treatment. This study shed the light to development of treatments to PSD patient in the future.

Abbreviation

FNS (fastigial nucleus electrical stimulation); PSD (post-stroke depression); rCBF (regional cerebral blood flow); MCAO (Middle cerebral artery occlusion); OFT (open field test).

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Disclosure Statement

The authors declare that there is no conflict of interest

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