Trigeminal neuralgia causes neurodegeneration in rats associated with upregulation of the CD95/CD95L pathway

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ABSTRACT

Objectives: To explore the effects of trigeminal neuralgia on neurodegeneration in rats and the underlying mechanism.

Methods: 60 adult male SD rats were divided randomly into Chronic Constriction Injury of the Rat's Infraorbital Nerve group (ION-CCI) and Sham group (n = 30). Right suborbital nerve was ligated in ION-CCI group to establish a trigeminal neuralgia model. In sham group, suborbital nerve was exposed without ligation. Pain thresholds were measured before surgery, 1, 7, 15, and 30 days after surgery (n=10). Morris water maze tests (n=10) were conducted 1, 15 and 30 days after surgery to evaluate the changes in learning and memory ability of rats. 5, 19, and 34 days after surgery, serum S100β protein concentration and hippocampal Aβ1-42 protein expression were detected by ELISA, Total Tau protein expression was detected by Western blotting, changes of neurons in hippocampus observed by Nissl staining, and the expression of p-ser404-tau, CD95, CD95L, and Cleaved caspase-3 proteins were detected by immunofluorescence and Western blotting.

Results: Hyperalgesia occurred in ION-CCI group and mechanical pain threshold decreased significantly (P<0.05). On the 15th and 30th days after surgery, ION-CCI group showed lower learning and memory ability.
than Sham group (P<0.05). Serum S100β protein concentration, hippocampal Aβ1-42 and ser404p-tau protein expression increased in the ION-CCI group 19 and 34 days after surgery (P<0.05), hippocampal CD95 expression increased in the ION-CCI group after surgery (P < 0.05), hippocampal CD95L expression increased at 19 and 34 days after surgery (P < 0.05), and Cleaved caspase-3 expression increased at 5 and 19 days after surgery (P < 0.05). Nissl bodies in ION-CCI group decreased significantly at 15 days after surgery. The expression of Cleaved caspase-3 protein in ION-CCI group was positively correlated with the expression of CD95 and CD95L (P < 0.05).

**Conclusions:** Trigeminal neuralgia may lead to neuronal inflammation and neuronal apoptosis associated with upregulation of CD95/CD95L expression, thus causing neurodegeneration.

**Keywords**

Trigeminal neuralgia; CD95/CD95L; neuroinflammation; cell apoptosis; neurodegeneration; hippocampus; cognitive dysfunction; Nissl staining
INTRODUCTION

Recent studies have found that patients with chronic pain are often accompanied by cognitive dysfunction, which seriously affects the quality of life of patients (1-3). Existing studies suggested that changes in hippocampal synaptic structure, changes in the expression of neurotransmitters (cytokines, brain-derived neurotrophic factors), and overuse of opioids might be the mechanisms of cognitive dysfunction in patients (4,3), but the exact mechanism remains to be further studied.

Trigeminal neuralgia is a typical clinical chronic neuropathic pain. Meskal I etc (5) suggested that patients with trigeminal neuralgia are at risk of cognitive defects. In addition, studies have reported that trigeminal neuralgia can damage spatial learning and memory ability of rats (6,7). The hippocampus is involved in the regulation of learning, memory, pain and emotional response, damage to the hippocampus can lead to impaired learning and memory (8-10), after nerve injury, the transmission of nociceptive stimulation to the central nervous system can lead to increased expression of cytokines in the hippocampus, such as TNF-α, IL-6, etc (11-14). These factors act on neurons, microglia or astrocytes, activate inflammatory responses, participate in the regulation of pain, and induce and maintain neuropathic pain (15-17). In addition, neuropathic pain also increased apoptosis of hippocampal neurons in rats (18,19). These evidences suggest that hippocampus is closely related to cognitive impairment in neuropathic pain animal models.
Among the related diseases with cognitive impairment as the main symptom, neurodegenerative disease is the most typical and common, presenting as progressive cognitive dysfunction. Neuroinflammation and neuronal apoptosis are important mechanisms of neurodegenerative disease, among which Alzheimer's disease is more common. Amyloid β-protein (Aβ) deposits form Senile plaques (SPs) and Neurofibrillary tangles (NFTs), which are formed by hyperphosphorylation of Tau protein. CD95 expression was up-regulated in patients with Alzheimer's disease (20,21), it is suggested that CD95 is closely related to neurodegeneration, but whether CD95 is related to trigeminal neuralgia is not clear at present.

CD95 is type I transmembrane protein, known as "death receptors", CD95L is type II transmembrane protein, it is the ligand of CD95, both belong to the tumor necrosis factor superfamily member. It was found that CD95/CD95L played an important role in the development, regeneration and apoptosis of the nervous system (22,23), under physiological conditions, CD95/CD95L is only slightly expressed in the brain to maintain immunosuppression, while under pathological stimulation such as craniocerebral injury or stroke, CD95L may be highly expressed in neurons and glial cells. CD95/CD95L was found to promote the secretion of cytokines (24,25), while cytokines such as IL-6, IL-8, and IL-1β have been proved to be key factors in inducing neuroinflammatory responses, so CD95/CD95L can induce neuroinflammatory responses by promoting
the secretion of inflammatory cytokines. Meanwhile, CD95/CD95L can activate the cascade reaction of Caspase family and lead to apoptosis. Combined with the above studies, neuropathic pain can lead to neuroinflammatory response and neuronal apoptosis, and neuroinflammatory response and neuronal apoptosis play an important role in neurodegeneration. Therefore, we hypothesized that CD95/CD95L pathway may be involved in the pathogenesis of cognitive impairment caused by trigeminal neuralgia.

Based on the above results, this study intends to explore whether trigeminal neuralgia affects the neurodegeneration of rats through the CD95/CD95L pathway in the model rats of trigeminal neuralgia.

METHODS

Animals

67 healthy and clean adult male SD rats weighing 200-250g were used in this experiment, provided by animal experiment center of Southwest Medical University. All animal procedures were approved by the Ethical Committee of Southwest Medical University, Luzhou, China. Additionally, all animal procedures in the experiment in strict accordance with the regulations of the People's Republic of China on the management of experimental animals and the methods for the quality management of experimental animals.

Three days before the experiment, the animals were trained to adapt the environment. The rats were knocked on the cage wall with a plastic
rod and the rats' tails were lifted. After the rats were calm, the whisker pads were stimulated with a self-made brush for three times on each side. The bilateral stimulation was carried out alternately until the rats changed from initial exploration, fear curling, nose whipping, rapid escape, aggression and other behaviors to silence. After 3 days of the above training, those who showed calmness and did not lose their whiskers were selected.

Rats were randomly divided into surgery group (ION-CCI group) and Sham group (Sham group), with 30 rats in each group and Sham group as the control group. ION-CCI group under the line of Chronic constriction of the inferior orbital nerve (Infraorbital nerve chronic constriction injury, ION-CCI) to establish model of trigeminal neuralgia, Sham group only exposed orbital nerve without ligation.

**Surgical Procedures**

Anaesthesia was given by intraperitoneal injection of sodium pentobarbital (40 mg/kg), a 1cm incision was made along the upper margin of the right palpat pad of the rat to separate and expose the infraorbital nerve (Fig. 1A), and then two chromium-enteric wires (4-0) were used to ligate the nerve at the proximal and distal ends, with an interval of about 2mm. The standard of ligation was that the diameter of the infraorbital nerve became slightly thinner, but the conduction was not completely blocked and the blood circulation of the nerve outer membrane was unobstructed to prevent nerve ischemia and necrosis, and
the incision was closed after surgery. The suborbital nerve was exposed in
the Sham group by the same method without ligation.

**Behavioral Testing**

Mechanical pain threshold measurement: to evaluate whether the
establishment of trigeminal neuralgia model is successful.

Rats were placed in a mesh cage and waited until the rats were silent,
the test subjects used the Von Frey fiber filament to stimulate the rat’s
surgical side whiskers (Fig. 1B). Starting from the minimum stimulus
intensity of 0.008g, and gradually increasing, until the rats showed
withdrawal and avoidance, aggression, and asymmetric face scratching
behavior, it was considered as a positive reaction. Each stimulus intensity
was tested 3 times/side, and when the positive number of the 3 stimuli
was 2, the corresponding fibrofilament strength was the mechanical pain
threshold of the rat. If the stimulation intensity was 26g and the rats still
did not have the above positive reaction, the test was stopped and 26g
was taken as the threshold value of this test. The test time included 5 time
points: before surgery, 1, 7, 15, and 30 days after surgery. At each time point,
two groups of rats were randomly selected for pain threshold
measurement (n=10).

Morris water maze: test of learning and memory ability

Morris water maze consists of a black circular pool (160 cm
diameter, 50 cm height), a cylindrical platform (12 cm diameter) and a
computer camera analysis system. The water pool was covered by blue floodlight curtains and divided into four quadrants. Different markers were placed in each quadrant to provide visual clues for the rats to find the platform. In one quadrant middle place escape platform, platform in 2 cm below the surface, pool water temperature at 20℃-22℃, for rat to provide a stable environment so as to avoid external factors interfere with the behavior of rats.

The rats trained continuously for 4 days, 4 times a day, 30 seconds apart. In each training, the rats face the inner wall of the pool and were put into the pool from the midpoint of the wall of each quadrant. The time for the rats to stand on the escape platform was the result of the time. If the platform was not found within 120 seconds, it will be recorded as 120 seconds. The average of 4 training sessions per day was used as the day's escape latency. On day 5, the platform was removed and the rats were put into the pool from the opposite quadrant of the original escape platform. The number of times the rats crossed the original platform and the percentage of time they spent in the target quadrant in 120 seconds were recorded.

1,15, and 30 days after the surgery, the rats after the pain threshold measurement were subjected to the water maze experiment, which were recorded as Test 1, Test 2, and Test 3, respectively.
Sampling and Sample Preparation

After the completion of Test 1, Test 2 and Test 3 of the water maze experiment, namely 5 days, 19 days and 34 days after the operation, rats were sacrificed and samples were obtained. At three time points respectively from two groups of rats (n = 5) were randomly selected with sodium pentobarbital (40 mg/kg) abdomen anesthesia, after exposure to the heart and inferior vena cava, 3 to 4 ml of vena cava blood samples were kept for 1 hour, then centrifuged (3000 r / min 10 min) to separate the serum, serum after stored in light suck out -80℃ refrigerator for later use. Cardiac perfusion with 0.1m phosphate buffer saline（PBS;pH7.4）, then perfusion precooling of 4% paraformaldehyde was performed before tissue fixation. The severed head was removed and the brain was fixed in 4% paraformaldehyde for 24 h, then through dehydration, paraffin, embedding, using paraffin section of the machine line continuous coronary slice, 3 microns thick, at 60℃ oven for the night, out put at room temperature after saved for later use. Only five other rats with PBS perfusion, and then immediately cut off the head on ice to get the hippocampus, after the tissue homogenate under 4℃ to 13000 r/min, the centrifugal 15 minutes, the supernatant was removed and the protein concentration was measured by BCA method, supernatant fluid is used in ELISA to detect Aβ1-42 protein and Western Blotting.

ELISA: the concentration of S100β protein and the expression of
Aβ1-42 protein in the hippocampus of rats were detected

Remove the ELISA Kit (Rat S100βELISA Kit, Rat Aβ1-42 ELISA Kit, qiaodu biotechnology, Shanghai, China) and balance at room temperature for 20 min. Standard sample and sample hole in turn add, then each hole in horseradish peroxidase (HRP) labeled detection antibody, sealing plate and temperature of 37°C) 60 minutes, wash the plate after joining the substrate avoid light incubation 15 minutes, add terminated liquid, measuring OD value.

Western Blotting: The expression of CD95, CD95L, Cleaved caspase-3, Total Tau and ser404p-tau in hippocampal tissues were detected

SDS-PAGE was used to isolate the equal amounts of protein and transfer it to PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk at room temperature, but ser404p-tau was blocked with 5% BSA. Then, with a primary antibody under 4°C overnight incubation (rabbit anti CD95, 1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA. Rabbit anti-CD95L, 1:400, rabbit anti-cleaved caspase-3, 1:1000, rabbit anti-total Tau, 1:1000, rabbit anti-ser404p-tau, 1:1000, Abcam, Cambridge, MA, UK). Then, the membranes were incubated with anti-rabbit secondary antibody (1:500, Proteintech, Chicago, IL, USA) at room temperature for 1 hour. Finally, the bands were detected using ECL (Beyotime, Shanghai, China) chemiluminescence. Image J (National Institutes of Health, Bethesda, MD, USA) was used for quantitative band intensity; GAPDH (1:500, Abcam, Cambridge, MA, UK) was used as the sample control.

Immunofluorescence Test: expressions of CD95, CD95L, and Cleaved caspase-3 proteins were detected
After section dewaxing and gradient ethanol rehydration, wash with distilled water. The sections were then placed in an antigen repair solution (Beyotime, Shanghai, China), incubate in a water bath at medium temperature for 20 minutes. After natural cooling to room temperature, remove the sections and break the membrane in 0.2% Triton X-100 solution (Amresco, USA) for 15 minutes. Seal the sections in 1% BSA for 30 minutes. Then with a primary antibody incubation (CD95:1:50, CD95L: 1:100, Cleaved - caspase3:1:200) under 4°C for the night. Incubate with different fluorescent-labeled secondary antibodies (CY3:1:200, FITC: 1:100, CWBIO, Beijing, China) at dark and room temperature for 1 hour. DAPI (Beyotime, Shanghai, China) was stained for 5 minutes. View the hippocampus under an optical microscope (Olympus, Tokyo, Japan) (magnification 400).

**Nissl staining: observing the changes of neurons in hippocampus**

Paraffin slices were dewaxed to water routinely, then reagent A (Methylene Blue Stain) was dripped for 10 minutes, and then differentiated into reagent B (Nissl Differentiation). The slices were observed under a microscope until the Nissl body was clear. Next, reagent C (Ammonium Mordate Solution) was added to treat the slices for 4 minutes. The slices were rinsed with distilled water and dehydrated by anhydrous ethanol, xylene transparent, neutral resin blocked.
Statistical Analysis

SPSS 19.0 statistical software was used. Experimental data were expressed as mean ± standard deviation (χ ± s). Escape latency, swimming speed using repeated measures ANOVA, we used one-way ANOVA to compare the pain threshold, Crossing the original target platform times, target quadrant time ratio, protein expressions of CD95, CD95L, Cleaved - caspase3, S100 β, A β 1-42, Tau protein of different time points within the group, and LSD was used for pairwise comparison, comparison between group by t test, analysis using Pearson correlation analysis. P < 0.05 was considered statistically significant.

RESULTS

Changes of mechanical pain threshold in rats

The pain threshold of ION-CCI group rats was higher one day after surgery than that before surgery (P<0.05), and the subsequent time points were significantly lower than that before surgery (P<0.01). This abnormal sensitivity to pain persisted during the 30-day observation, especially at the 15th day after surgery. The sham group showed no such change (Fig. 2).

Results of rat Morris water maze experiment

In order to compare the effects of trigeminal neuralgia on cognitive function, we conducted three water maze experiments to observe the behavioral changes of rats, respectively Test 1, Test 2 and Test 3. Each
experiment included four consecutive days of positioning and navigation training and the fifth day of space exploration test. With the prolongation of learning time, the escape latency of rats was gradually shortened. In Test 1, there was no significant difference between the two groups in escape latency ($F = 0.153, P = 0.701$) (Fig. 3A) and swimming speed ($F = 1.553, P = 0.229$) (Fig. 3D), that meant, 1 day after surgery, the learning function of the rats was not significantly impaired. In Test 2, the escape latency of ION-CCI group rats was significantly longer than that of Sham group ($F = 101.315, P = 0.000$) (Fig. 3B). There was no statistical difference in swimming speed ($F = 0.485, P = 0.495$) (Fig. 3E), suggesting that trigeminal neuralgia impaired learning ability 15 days after surgery. In Test 3, the escape latency of ION-CCI group rats was longer than that of Sham group ($F = 95.984, P = 0.000$) (Fig. 3C), and the swimming speed showed no statistical difference ($F = 0.010, P = 0.923$) (Fig. 3F), suggesting that trigeminal neuralgia has sustained damage to learning ability. On the fifth day of training, after the rats were removed from the platform, the space exploration experiment was conducted on the rats. Compared with Sham group, the number of crossing the platform (Fig. 4A) and the time percentage in the target quadrant of ION-CCI group were significantly reduced ($P < 0.01$) (Fig. 4B), while there was no significant difference between the two groups in Test 1 (Fig. 4A, B).
Expression of neurodegenerative -related proteins was increased in trigeminal neuralgia rats

S100β protein is a specific protein of the nervous system, which is closely related to pain and cognitive function. Therefore, we used the ELISA to detect whether S100β protein in serum was up-regulated. The results showed that there was no statistically significant difference in the concentration changes at postoperative time points in the Sham group (F = 0.602, P = 0.555), while the ION-CCI group was significantly higher at 19 and 34 days after surgery than the Sham group (P < 0.05), with the highest concentration at 19 days after surgery (Fig. 5A). Excessive deposition of Aβ can lead to cognitive dysfunction. Therefore, we detected the expression of Aβ 1-42 protein in the hippocampus by ELISA. The results showed that there was no statistical difference in postoperative time points in the Sham group (F = 0.379, P = 0.692), and ION-CCI group was significantly higher than that in the Sham group at 19 and 34 days after surgery (P < 0.05), with the highest value at 34 days after surgery. (Fig. 5B). Tau protein phosphorylation level in the normal brain tissue is very low, when pathological changes occur, Tau protein can be over-phosphorylated at multiple sites, which affect cognitive function, therefore, we used the Western Blotting to detect the Tau protein expression in the hippocampus, the results showed two groups of rats with the change of the Total - Tau protein had no statistical difference
(P > 0.05), and ION - CCI group $^{\text{Ser}404} P$ - Tau protein in hippocampus of rats with Aβ1-42 as rising trend, compared with the Sham group, there was a significant increase at 19 and 34 days after surgery (P < 0.05), with the highest increase at 34 days after surgery (Fig. 5C, D).

**The expression of CD95/CD95L pathway was up-regulated in the hippocampus of trigeminal neuralgia rats**

Previous clinical studies have shown that CD95 plays an important role in neurodegenerative diseases, such as Alzheimer's disease. Therefore, we detected whether the CD95/CD95L pathway was associated with cognitive impairment induced by trigeminal neuralgia. We detected the expression of CD95, CD95L, and Cleaved caspase-3 in the hippocampus of rats 5, 19, and 34 days after surgery by immunofluorescence and Western Blotting.

Immunofluorescence results showed that the green fluorescence expression of CD95 and CD95L protein and the red fluorescence expression of Cleaved caspase-3 protein were weak in the Sham group, while the fluorescence expression of ION-CCI group was obvious (Fig. 6A,B,C). Quantitative detection of hippocampal CD95, CD95L, and Cleaved caspase3 protein expression by Western Blotting showed that no significant differences were found in CD95 (F = 0.496, P = 0.621), CD95L (F = 2.054, P = 0.171), and Cleaved caspase3 (F = 2.463, P = 0.127) expressions at various time points in the Sham group. CD95
expression in ION-CCI group was significantly increased at each time point after surgery (P < 0.05), with the highest expression at 19 days after surgery (Fig. 7A, D). CD95L showed a similar trend to that of CD95, which was significantly higher at 19 and 34 days after surgery than in the Sham group (P < 0.05), with the highest expression at 19 days after surgery (Fig. 7B, E). Cleaved caspase-3 expression was significantly higher at 5 and 19 days after surgery than in the Sham group (P < 0.05), with the highest expression at 19 days after surgery (Fig. 7C, F). We also measured the expression level of CD95, CD95L, Cleaved caspase-3, Total Tau and ser404p-tau proteins in prefrontal cortex. To our surprise, we did not observe the same change trend in prefrontal cortex as in hippocampus, and they did not show a stable and consistent trend of change. (data not shown)

The Cleaved caspase-3 protein in hippocampus of trigeminal neuralgia rats was correlated with CD95/CD95L protein

The expression of Cleaved caspase-3 protein in the hippocampal tissues of ION-CCI rats was positively correlated with the expression of CD95 protein (r = 0.570, P = 0.027) (Fig. 8A), and the expression of Cleaved caspase-3 protein was positively correlated with the expression of CD95L protein (r = 0.650, P = 0.009) (Fig. 8B).

Changes of neurons in hippocampus

In Sham group, the structure of neurons in CA3 area of hippocampus
was intact, nucleolus was obvious, and a large number of granular and tigroid Nissl bodies could be seen in the cytoplasm (Fig. 9A). While in ION-CCI group, the neurons in CA3 area of hippocampus were mostly vacuolar, with no obvious nucleolus, and Nissl bodies dissolved or even disappeared (Fig. 9B).

**DISCUSSION**

Recent studies have found that head and facial pain is closely related to cognitive dysfunction (26-30), while trigeminal neuralgia is the most common chronic neuropathic pain in the head and face, its pathogenesis is complex, the current mainstream is divided into central and peripheral mechanism, the central theory of trigeminal neuralgia associated with voltage channel gating, and around the theory of trigeminal nerve demyelination changes cause "short circuit" between adjacent nerves, "short circuit" phenomenon of abnormal nerve impulses to the central cause sustained excitement. In order to simulate different pathogenesis, researchers have successfully established a variety of animal models of trigeminal neuralgia, including ION-CCI model, dental pulp perfusion model and suborbital nerve injection model of cobra venom. This study is to replicate Liu CY etc. (31) through the zygomatic line down to the road of ION - CCI model of trigeminal neuralgia. ION-CCI group rats showed a low response period one day after surgery, which may be due to axonal degeneration after nerve ligation, and the nerve impulse could not be
transmitted through the ligation site, resulting in increased pain threshold. However, the specific mechanism remains to be further studied. The pain threshold was the lowest 15 days after surgery, which was considered to be the most severe myelin damage during this period. The pain threshold was slightly increased 30 days after surgery, this may be due to gradual absorption of the catgut and the nerve was gradually repaired. This is consistent with the results reported in previous studies, indicating that the modeling is successful.

In this study, the Morris water maze experiment was conducted at three time points (1 day, 15 days and 30 days after the surgery) from the acute stage, pain sensitive stage and chronic stage after nerve injury in combination with the aforementioned changes in pain threshold, to verify that chronic trigeminal neuralgia would lead to spatial learning and memory dysfunction in rats. However, we found that at 30 days after surgery, the learning and memory ability of rats showed an opposite trend to the change of pain threshold, which may be due to the gradual recovery of the injury of the infraorbital nerve after catgut absorption, but the effect of pain on the central nervous system did not recover. Previous studies often choose to test the water maze behavior at a point in time, the data are relatively simple, and this experiment observed the three time points, more fully reflect the dynamic change of learning and memory ability of rats, so this study can reflect the trend of trigeminal neuralgia
effect on cognitive function. However, whether the learning and memory abilities continue to decline or improve after 30 days remains to be further explored.

S100β protein is a specific protein of the nervous system. When the central nervous system is injured, S100β protein exudes from the damaged site to the cerebrospinal fluid, and then enters the blood through the blood-brain barrier. In addition, studies have reported increased serum S100β protein concentration in AD patients (32), therefore, serum S100β protein is often used as an indicator of the extent of reactive nerve injury and the prediction of neurodegeneration. The results of this study are consistent with previous studies showing that pain can lead to the upregulation of S100β protein levels (33-35). In particular, serum S100β protein levels were significantly up-regulated 19 and 34 days after surgery, suggesting severe nerve damage in rats during this period. At the same time, the upregulation of S100β protein also suggested neurodegeneration in rats.

An overdeposit of Aβ is an important pathology feature of AD, Aβ includes Aβ1-40 and Aβ1-42, Aβ1-42 because it's hydrophobic and it's much more likely to be the main ingredient in the SPs, so this study is to test the expression of Aβ1-42. Abnormal phosphorylation of Tau protein can reduce the binding ability of Tau protein to microtubules, the combination of Tau protein and microtubule cytoskeleton damage,
leading to the formation of a NFTs, thus occurring nerve degeneration, Ser404 is considered to be a reliable marker of the transition from mild cognitive impairment to AD, moreover, phosphorylation of this site was crucial for the formation of Paired helical filaments (PHF), so this study selected Ser404 site to detect the Tau protein phosphorylation level. In our study, Aβ1-42 and Ser404-P-Tau level are raised after surgery, prompt rats nerve degeneration, may have happened when the pain threshold began to recover gradually, Aβ1-42 and Ser404-P-Tau level is still on the rise, the tip of the central nervous system may be irreversible damage, its mechanism and the in between is Aβ and P-Tau synergies related to each other (36,37), further research is needed to confirm this.

S100β protein, Aβ protein and p-tau protein are neurodegenerative protein-related proteins. The expression of all three was up-regulated in the hippocampus of trigeminal neuralgia rats, in combination with the water maze rats behavior change, further confirmed the trigeminal neuralgia can cause nerve degeneration assumption, this is consistent with previous studies that neuropathic pain can lead to cognitive dysfunction, in this study, we further explore its possible mechanism.

Recent studies have demonstrated the importance of the CD95/CD95L pathway to the nervous system, which is involved in the occurrence and development of a variety of neurological diseases such as Alzheimer's disease (38-41), but whether it is associated with neuropathic
pain is not clear. The main function of CD95/CD95L is to participate in the immune response, etc. And a number of studies suggest that pain may affect the immune response of the body (42,44). In this study, we found that trigeminal neuralgia resulted in up-regulation of CD95/CD95L expression in the hippocampus of rats. The processing of pain information by the nervous system needs to be completed through a complex neural network. The upregulation of CD95/CD95L induced by trigeminal neuralgia may be related to the upward conduction of pain information. When pain information from the periphery is transmitted to the central nervous system, it may activate the hippocampus to participate in the processing of pain information, but the exact mechanism remains to be determined by further research.

The prefrontal cortex is also involved in the regulation of spatial learning and memory abilities, but in our study, no up-regulation of these proteins was found in the prefrontal cortex, which suggests that CD95/CD95L pathway may be one of the molecules specifically expressed in the hippocampus in cognitive impairment caused by trigeminal neuralgia. Although hippocampus and prefrontal cortex have many similar functions, their specific molecules may not be consistent. Studies have shown that in RHI rats with poor learning and memory ability and cognitive impairment, some synaptic related molecules show different trends in hippocampus and prefrontal cortex (45). Of course,
whether there are also molecular pathways related to cognitive impairment caused by trigeminal neuralgia in hippocampus can be the direction of our future research.

Upregulation of CD95/CD95L may affect the occurrence of neurodegeneration through multiple pathways. It can promote the secretion of cytokines such as IL-6, IL-8, and IL-1β or the migration of immune cells such as neutrophils to cause neuroinflammatory reactions, which are caused by long-term chronic neuroinflammatory reactions. At the same time, CD95 is also a signaling molecule upstream of the Caspase apoptosis pathway, which activates the cascade reaction of the Caspase family after binding to CD95L, among which the activation shearing of Caspase3 is the most critical, and eventually leads to the apoptosis of cells due to the degradation of DNA. Cleaved caspase-3 is an iconic protein of cell apoptosis. The increased expression of Cleaved caspase-3 protein after ION-CCI surgery suggested that trigeminal neuralgia increased the apoptosis of hippocampal cells, which was consistent with the results of previous animal studies that pathological pain increased the apoptosis of hippocampal cells in rats (8,19). Abnormal increase of apoptosis leads to excessive loss of neurons, which can lead to neurodegeneration. This study found that the expression of Cleaved caspase-3 protein was positively correlated with the expression of CD95/CD95L, suggesting that the CD95/CD95L apoptosis pathway
might be involved in the apoptosis of hippocampal cells in rats with trigeminal neuralgia. Secondly, CD95/CD95L can affect Aβ and Tau proteins by stimulating the activation of downstream Caspase3, and studies have found that Caspase3 can regulate the production of Aβ (46), specific mechanism is: γ-secretase hydrolysis APP generates Aβ, γ-secretase activating protein (γ-secretase - activating protein, GSAP) is the key to adjust the process protein, and GASP is regulated by Caspase3, so after Caspase3 activation can promote the formation of Aβ, and activation of Caspase3 can also cut the Tau protein, exposed Tau protein phosphorylation sites, easier to aggregate.

Nissl bodies exist in neuron bodies or dendrites and are basophilic granules. It can reflect the changes of morphological structure and function of neurons (47). In this study, the obvious histological changes of neurons in rats with trigeminal neuralgia were observed by Nissl staining, which is another direct and strong evidence that trigeminal neuralgia causes neurodegeneration in rats.

In this study, the above experiments indicated that trigeminal neuralgia may lead to increased neuroinflammation and neuronal apoptosis by up-regulating the expression of CD95/CD95L, thus causing neurodegeneration. However, further studies are needed to determine the exact mechanism of up-regulation of CD95/CD95L.

CONCLUSIONS
This study verified that trigeminal neuralgia can cause learning and memory dysfunction in rats, and revealed that the upregulation of the hippocampal CD95/CD95L pathway may be involved in relevant mechanisms, providing a theoretical basis for the prevention and intervention of cognitive dysfunction in patients with trigeminal neuralgia.

Limitations

Trigeminal neuralgia causes neurodegeneration in rats associated with upregulation of the CD95/CD95L pathway, but the up-stream impact factors of this pathway remains to be elucidated. The exact mechanism of the CD95/CD95L pathway needs to be further determined using knockout rats.
Declaration of Conflicting Interests

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Figure 1. (A) Surgical procedures: After anaesthesia by intraperitoneal injection of 10% chloral hydrate (4 ml/kg), a 1 cm incision was made along the upper margin of the right palpal pad of the rat to separate and expose the infraorbital nerve; (B) Mechanical pain threshold measurement: The rat was placed using a reticulated rat cage and waited until the rats were silent, the test subjects used the Von Frey fiber filament to stimulate the rat's surgical side whiskers.

Figure 2. Change of the mechanical threshold in two groups. Mechanical threshold. Data are expressed as Mean ± SD. (#P < 0.05, compared to preoperative; *P < 0.05, compared to sham group).
Figure 3. Learning ability in rats on the Water maze experiment. (A) Escape latency in Test 1 ($F=0.153, P=0.701$); (B) Escape latency in Test 2 ($F=101.315, P=0.000$); (C) Escape latency in Test 3 ($F=95.984, P=0.000$); (D) Swimming speed in Test 1 ($F=1.553, P=0.229$); (E) Swimming speed in Test 2 ($F=0.485, P=0.495$); (F) Swimming speed in Test 3 ($F=0.010, P=0.923$). Data are expressed as Mean ± SD. (*$P<0.05$, compared to sham group).

Figure 4. Probe trials in rats on the Morris water maze task. (A) Number of crossing the platform; (B) Time percentage in the target quadrant ($P < 0.01$). Data are expressed as Mean ± SD. (*$P<0.05$, compared to sham group).
Figure 5. Neurodegenerative-related proteins in trigeminal neuralgia rats. (A) serum S100β protein level; (B) hippocampus Aβ 1-42 protein level; (C) hippocampus Tau protein level; (D) hippocampus total Tau protein and Ser404P - Tau protein level.
Figure 6. Expression of CD95 protein, CD95L protein and Cleaved-caspase3 protein in hippocampus of Rats. (A) CD95 protein (The green fluorescence represents the CD95 protein, the blue fluorescence represents DAPI, 400×); (B) CD95L protein (The green fluorescence represents the CD95L protein, the blue fluorescence represents DAPI, 400×); (C) Cleaved-caspase3 protein (The red fluorescence represents the Cleaved-caspase3 protein, the blue fluorescence represents DAPI, 400×).
Figure 7. Quantitative detection of hippocampal CD95, CD95L, and Cleaved Caspase3 protein at various time points. (A) (D) CD95 expression; (B) (E) CD95L expression; (C) (F) Cleaved caspase-3 expression.

Figure 8. Correlation between Cleaved-caspase3 protein and CD95/CD95L protein level in hippocampus in ION-CCI group. (A) The expression of Cleaved-caspase3 and CD95 were positively related (r=0.570, P=0.027); (B) the expression of Cleaved-caspase3 and CD95L were positively related (r=0.650, P=0.009); P<0.05, there is statistical significance.
Figure 9. Nissl staining: the changes of neurons in hippocampus. (A) Morphology of hippocampal neurons in Sham group; (B) morphology of hippocampal neurons in ION-CCI group.