Stimulation of Anterior Thalamic Nuclei Protects Against Seizures and Neuronal Apoptosis in Hippocampal CA3 Region of Kainic Acid-induced Epileptic Rats

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Background: The antiepileptic effect of the anterior thalamic nuclei (ANT) stimulation has been demonstrated; however, its underlying mechanism remains unclear. The aim of this study was to investigate the effect of chronic ANT stimulation on hippocampal neuron loss and apoptosis.

Methods: Sixty-four rats were divided into four groups: The control group, the kainic acid (KA) group, the sham-deep brain stimulation (DBS) group, and the DBS group. KA was used to induce epilepsy. Seizure count and latency to the first spontaneous seizures were calculated. Nissl staining was used to analyze hippocampal neuronal loss. Polymerase chain reaction and Western blotting were conducted to assess the expression of caspase-3 (Casp3), B-cell lymphoma-2 (Bcl2), and Bcl2-associated X protein (Bax) in the hippocampal CA3 region. One-way analysis of variance was used to determine the differences between the four groups.

Results: The latency to the first spontaneous seizures in the DBS group was significantly longer than that in the KA group (27.50 ± 8.05 vs. 16.38 ± 7.25 days, P = 0.0005). The total seizure number in the DBS group was also significantly reduced (DBS vs. KA group: 11.75 ± 6.80 vs. 23.25 ± 7.72, P = 0.0002). Chronic ANT-DBS reduced neuronal loss in the hippocampal CA3 region (DBS vs. KA group: 23.58 ± 6.34 vs. 13.13 ± 4.00, P = 0.0012). After chronic DBS, the relative mRNA expression level of Casp3 was decreased (DBS vs. KA group: 1.18 ± 0.37 vs. 2.09 ± 0.46, P = 0.0003), and the relative mRNA expression level of Bcl2 was increased (DBS vs. KA group: 0.92 ± 0.21 vs. 0.48 ± 0.16, P = 0.0004). The protein expression levels of CASP3 (DBS vs. KA group: 1.25 ± 0.26 vs. 2.49 ± 0.38, P < 0.0001) and BAX (DBS vs. KA group: 1.57 ± 0.49 vs. 2.80 ± 0.63, P = 0.0012) both declined in the DBS group whereas the protein expression level of BCL2 (DBS vs. KA group: 0.78 ± 0.32 vs. 0.36 ± 0.17, P = 0.0086) increased in the DBS group.

Conclusions: This study demonstrated that chronic ANT stimulation could exert a neuroprotective effect on hippocampal neurons. This neuroprotective effect is likely to be mediated by the inhibition of apoptosis in the epileptic hippocampus.

Key words: Anterior Thalamic Nuclei; Apoptosis; Deep Brain Stimulation; Epilepsy; Hippocampus

INTRODUCTION

Temporal lobe epilepsy (TLE) is the most common type of focal epilepsy and is frequently resistant to drug treatment. Hippocampal sclerosis (HS), which is characterized by severe neuronal loss and gliosis in the hippocampus, is the prominent histopathological finding in TLE.1-3 Several factors, including febrile seizures, inflammation, brain trauma, and status epilepticus, may contribute to HS.1,3,4 It has been suggested that neuronal apoptosis constitutes one of the central mechanisms of neuronal death in the sclerotic hippocampus.5-8 Recent studies have revealed that neuronal apoptosis also has an important role in epileptogenesis.9 Considering the gradual neuronal apoptosis in the sclerotic hippocampus during the epileptogenesis process, the inhibition of
neuronal apoptosis in the hippocampus may become a novel approach in the treatment of epilepsy.

The antiepileptic effects of stimulation of the anterior nucleus of the thalamus (ANT) have been demonstrated by clinical trials and animal studies.[10-13] Many epilepsy patients who are not candidates for epilepsy surgery or continue to have seizures after epilepsy surgery may benefit from ANT-deep brain stimulation (DBS). Recently, we have shown that ANT stimulation induced favorable modulation in neurotransmitter levels by inhibiting the hyperactivation of excitatory pathways and promoting the inhibitory pathways.[14,15] These findings indicate that ANT stimulation may inhibit neuronal overexcitation in epilepsy and may exert neuroprotective effects. It is also reported that hippocampal stimulation could protect hippocampal neurons by inhibiting neuronal apoptosis.[16] These interesting findings illustrate that ANT stimulation may also have anti-apoptotic effects. In this study, we investigated the anti-apoptotic effects of ANT stimulation on the hippocampus in kainic acid (KA)-induced epileptic rats.

**Methods**

**Animals**

Sixty-four adult male Sprague-Dawley rats (Vital River Laboratories, Beijing, China), weighing 250–300 g, were used in this experiment. The rats were divided into four groups: (1) the control group (n = 16, rats only received saline injection); (2) the KA group (n = 16, rats only received KA injection); (3) the sham-DBS group (n = 16, rats received KA injection and electrodes implantation but without stimulation); and (4) the DBS group (n = 16, rats received KA injection, electrode implantation, and stimulation). The rats were raised in a common animal cage in an environmentally controlled room (20–23°C, 12-h light/12-h dark cycle, lights on at 7 a.m.) and given free access to food and water. All animal experiments were performed in accordance with the Guidance for Animal Experimentation of the Capital Medical University and Beijing guidelines for the care and use of laboratory animals.

**Stereotactic procedures**

Rats were anesthetized with 10% chloral hydrate (0.3 ml/kg) intraperitoneally and mounted in a stereotaxic frame (David Kopf Instruments, USA). Two guider canulas (RWD Life Science, China) were implanted in the left ANT (anterior-posterior [AP] = −1.4 mm, mediolateral [ML] = −1.2 mm, dorsoventral [DV] = 4.0 mm) and left hippocampus (AP = −5.6 mm, ML = −4.5 mm, DV = 3.5 mm) in the rats of sham-DBS and DBS groups. The other rats were implanted with one guider cannula in the left hippocampus. The canulas were fixed by affixing dental acrylic to stainless steel screws that were fastened on the skull.

**Establishment of epileptic rats and electrical stimulation**

After one week of recovery, the guider styllet in the left hippocampus was replaced with a stainless steel injection cannula (2 mm below the tip of the guider cannula). KA (Sigma Aldrich, USA), at a dose of 0.6 µg (1 µg/µl), was injected in the left hippocampus. The injection was conducted with a 1 µl Hamilton microsyringe and persisted for 5–10 min. Control rats received an injection of normal saline instead. One day after KA injection, the guider styllet in the left ANT was replaced with a bipolar electrode (outer diameter 250 µm, inner diameter 50 µm, CBCRE30, FHC, USA) in the sham-DBS and DBS groups. The tip of the electrode was 2 mm below the tip of the cannula. Electrical stimulation was delivered using a pulse stimulator (Master 8, AMPI, Israel) in the DBS group. The parameters were 500 µA, 130 Hz, and 60 µs, according to our previous research.[17] Stimulation was delivered for two months. All rats were continuously monitored for behavioral seizures, with video recording in the cages. The severity of behavioral seizures was scored according to a five-point scale[17] as follows: Score 0: no response; Score I: ear and facial twiching; Score II: myoclonic jerks without rearing; Score III: myoclonic jerks, rearing; Score IV: turnover into side position, clonic-tonic seizures; and Score V: turnover into back position, generalized tonic-clonic convulsions. The latency from the injection to the first spontaneous seizures and the number of partial seizures (PSs, Scores I–III), and generalized seizures (GSs, Scores IV and V) were analyzed.

**Tissue collection**

In each group, eight rats were used for Western blotting and polymerase chain reaction (PCR) analyses. The other half was used for Nissl staining analysis. At the end of the stimulation, rats were deeply anesthetized. The rats used for Nissl staining were perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer solution, and the brains were removed. After postfixed for 24 h, the brains were embedded with paraffin. The rats used for Western blotting and PCR analyses were decapitated. The CA3 region of the hippocampus was dissected from the brain on ice, as previously reported.[18] Then, the tissues were placed into liquid nitrogen in freezing tubes for storage and use. The mRNA expression of caspase-3 (Casp3), Bcl2-associated X protein (Bax), and B-cell lymphoma-2 (Bcl2) were detected with PCR. The protein levels of CASP3, BAX, and BCL2 were detected by Western blotting.

**Nissl staining**

The paraffin embed brains were cut using a microtome. The coronal sections (5 µm) were stained with Cresyl violet (Sigma-Aldrich, USA) solution. In each animal, six sections were used for quantitative analysis. The neurons in the CA3 zone of the hippocampus were analyzed. We calculated the average quantity of neurons at the same site of all sections from each rat. The sections were observed and mounted under a light microscope (Olympus America Inc., USA). The data were represented as the number of cells per optical field (original magnification, ×400).

**Quantitative polymerase chain reaction**

Total RNA was isolated using the Trizol method according to the manufacturer’s protocol (Invitrogen, USA). Genomic DNA was removed using the RQ1 RNase-Free DNase (Promega, USA). The RNA was reverse transcribed
using a set of primers and Goscript Reverse Transcription System (Promega, USA). The primer sequences were as follows: β-actin: forward, 5'-CCCGCGAGTACAACCTTCT-3', reverse, 5'-CGTCATCCATGGCGAACT-3'; Casp3: forward, 5'-CCGACTTCTCTGATGCTTCTA-3', reverse, 5'-CATGACCCCTGGATGCTTCTA-3'; Bcl2: forward, 5'-GTACCTGAACCGGCATCTG-3', reverse, 5'-GGGGCCATATAGTTCCACAA-3'; Bax: forward, 5'-GTGAGCGGCTGCTTGTCT-3', reverse, 5'-GTGGGGGTCCCGAAGTAG-3'. β-actin was used as an internal control transcript. Every quantitative PCR contained 20 mmol/L Tris/HCl, 50 mmol/L KCl, 4 mmol/L MgCl2, 0.2 mmol/L dNTPs, dimethyl sulfoxide (1:20), SYBR Green (1:50,000), 0.02 U/µl Taq DNA polymerase, and 2 pmol/L each primer. The reaction conditions were set as follows: an initial denaturation for 3 min at 95°C; followed by 50 cycles of denaturing at 95°C for 3 s, annealing at 51°C for 15 s and extension at 72°C for 30 s. We evaluated every sample in triplicate.

**Western blotting analysis**

For protein extraction, tissue was homogenized in lysis buffer with protease inhibitor mixture. The lysates were incubated and centrifuged, and the supernatant was collected for analysis. As internal control, β-actin. The optical density of the intensity of the bands was performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). The membranes (Immobilon-PSQ membrane; Millipore Corp., USA) were blocked with 5% nonfat milk in Tris buffered saline containing Tween 20 (TBST) and then incubated with a rabbit anti-BCL2 polyclonal antibody (Abcam, USA), a rabbit anti-BAX polyclonal antibody (Abcam, USA) and a rabbit anti-CASP3 monoclonal antibody (Cell Signaling Technology, USA) overnight at 4°C. After washing with 0.1% TBST, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Lab., West Grove, PA, USA) for 1 h at room temperature and washed in 0.1% TBST solution. Bands were imaged with a ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA). The optical density of the intensity of the bands was performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

All data were expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with the Tukey’s *post hoc* test was used to determine whether different values of each group were statistically significant. All data were analyzed with SPSS 18.0 for windows (SPSS Inc., Chicago, IL, USA). A probability of P < 0.05 was considered statistically significant.

**RESULTS**

**Effects of anterior nucleus of the thalamus-deep brain stimulation on behavioral seizures**

After KA injection, the rats experienced status epilepticus within 60 min. No seizures were observed in the control group. The mean latency to the appearance of the first spontaneous seizures was 16.38 ± 7.25 days in the KA group and 17.25 ± 7.86 days in the sham-DBS group. There was a significant increase in the latency (27.50 ± 8.05 days) to the first spontaneous seizures in the DBS group (DS vs. KA group, q = 5.76, P = 0.0005; DBS vs. Sham-DBS group, q = 5.31, P = 0.0014) [Figure 1a]. Among the KA-treated rats without electrode implantation (KA group), the mean total spontaneous seizure number was 23.25 ± 7.72 (PSs, 20.25 ± 7.92; GSs, 3.00 ± 1.07). In the sham-DBS group, the total seizure number was 24.38 ± 7.85 (PSs, 20.75 ± 7.03; GSs, 3.63 ± 2.07). The DBS group experienced a significant reduction in the total seizure number (11.75 ± 6.80; DBS vs. KA group, q = 6.16, P < 0.0001; DBS vs. Sham-DBS group, q = 6.76, P < 0.0001) [Figure 1b]. The PSs number (10.88 ± 6.73) and GSs number (0.88 ± 0.83) were also significantly reduced in the DBS group (PSs: DBS vs. KA group, q = 5.17, P = 0.0019; DBS vs. Sham-DBS group, q = 5.45, P = 0.0011; GSs: DBS vs. KA group, q = 5.95, P = 0.0004; DBS vs. Sham-DBS group, q = 7.72, P < 0.0001).

**Effects of anterior nucleus of the thalamus-deep brain stimulation on the neuronal survival in the CA3 region of hippocampus**

The survival of neurons in the CA3 region of the hippocampus was detected by Nissl staining [Figure 2a]. The surviving neurons were significantly lower in the KA group (13.13 ± 4.00, q = 11.73, P < 0.0001) and sham-DBS group (12.13 ± 2.93, q = 12.30, P < 0.0001) than in the control group (33.65 ± 5.76). With two months of ANT stimulation, the number of surviving neurons in the DBS group (23.58 ± 6.34) was higher than that in the KA group (q = 5.97, P = 0.0012) and the sham-DBS group (q = 6.54, P = 0.0004) but lower than that in the control group (q = 7.57, P = 0.0019) [Figure 2b].

**Anterior nucleus of the thalamus-deep brain stimulation inhibit apoptosis in the CA3 region of hippocampus**

To investigate the hippocampal neuroprotective effect of ANT-DBS, the mRNA levels of Bax, Bcl2, and Casp3 in the CA3 region of the hippocampus were analyzed [Figure 3]. The levels of mRNA were normalized to that of the control group. Compared with the control group (Bcl2: 1.00 ± 0.21, Bax: 1.00 ± 0.31, Casp3: 1.00 ± 0.32), the expression of Bcl2 mRNA decreased in the KA group (0.48 ± 0.16, q = 7.75, P < 0.0001) and the sham-DBS group (0.40 ± 0.18, q = 8.92, P < 0.0001) [Figure 3a]. The expression of Bax and Casp3 increased in the KA group (Bax: 1.69 ± 0.36, q = 5.51, P = 0.0029; Casp3: 2.09 ± 0.46, q = 7.94, P < 0.0001) and the sham-DBS group (Bax: 1.78 ± 0.39, q = 6.18, P = 0.0008; Casp3: 2.22 ± 0.39, q = 8.91, P < 0.0001) [Figure 3b and 3c]. There was no significant difference in the levels of Bcl2, Bax, and Casp3 mRNA between the KA group and the sham-DBS group. After ANT stimulation, the epileptic rats experienced an up-regulation of Bcl2 (0.92 ± 0.21; DBS vs. KA group, q = 6.57, P = 0.0004; DBS vs. Sham-DBS group, q = 7.74, P < 0.0010) [Figure 3a]. The expression of Casp3 mRNA
significantly decreased in the DBS group (1.18 ± 0.37; DBS vs. KA group, $q = 6.65, P = 0.0003$; DBS vs. Sham-DBS group, $q = 7.61, P < 0.0001$) [Figure 3c]; however, the level of Bax mRNA remained high in the DBS group (1.66 ± 0.36; Figure 3c).
DBS vs. control group, \( q = 5.30, P = 0.0043 \); DBS vs. KA group, \( q = 0.21, P = 1.00 \); DBS vs. Sham-DBS group, \( q = 0.88, P = 0.92 \) [Figure 3b].

To verify these results, we further investigated the protein levels of BAX, BCL2 and proenzyme form of CASP3 in CA3 region of the hippocampus [Figure 4a]. The levels of these proteins were normalized to the control group. Compared with the control group (BCL2: 1.00 ± 0.27, BAX: 1.00 ± 0.45, CASP3: 1.00 ± 0.30), the level of CASP3 was elevated in the KA group (2.49 ± 0.38, \( q = 11.72, P < 0.0001 \)) and sham-DBS group (2.63 ± 0.46, \( q = 12.85, P < 0.0001 \)) [Figure 4b]. The level of BCL2 decreased in the KA group (0.36 ± 0.17, \( q = 7.46, P < 0.0001 \)) and the sham-DBS group (0.36 ± 0.15, \( q = 7.40, P < 0.0001 \)) [Figure 4c]. The relative BAX levels were 2.80 ± 0.63 in the KA group and 3.05 ± 0.72 in the sham-DBS group, which was higher than that in the control group (KA vs. control group, \( q = 8.77, P < 0.0001 \); sham-DBS vs. control group, \( q = 9.98, P < 0.0001 \)) [Figure 4d]. In the DBS group, ANT stimulation reduced the level of CASP3 (1.25 ± 0.26; DBS vs. KA group, \( q = 9.77, P < 0.0001 \); DBS vs. Sham-DBS group, \( q = 10.90, P < 0.0001 \)) and BAX (1.57 ± 0.49; DBS vs. KA group, \( q = 6.00, P = 0.0012 \); DBS vs. Sham-DBS group, \( q = 7.21, P < 0.0001 \)) [Figure 4b and 4d]. The level of BCL2 increased in the DBS group after 2 months of stimulation (0.78 ± 0.32; DBS vs. KA group, \( q = 4.91, P = 0.0086 \); DBS vs. Sham-DBS group, \( q = 4.85, P = 0.0095 \)) [Figure 4c].

**DISCUSSION**

In the present study, we observed significant neuronal loss and apoptosis in the CA3 region of the hippocampus in epileptic rats. With chronic unilateral ANT stimulation, it could not only reduce the incidence of KA-induced seizures in rats but also reduce apoptosis and neuronal loss in the epileptic hippocampus.

ANT is a relay structure in the Papez circuit and is correlated with the hippocampus through this circuit. In a multicenter, double-blind, randomized, and long-term follow-up study that reported the efficacy and safety of ANT-DBS for drug-resistant partial epilepsy, there was a 56% median percent seizure reduction at the 2nd year and a 69% median percent seizure reduction at the 5th year. The patients also experienced significant improvements in quality of life and no unanticipated adverse events. Electrophysiology studies have indicated that ANT stimulation could significantly reduce the hippocampal excitability and exert antiepileptic effects in epileptic rats.

Our study indicated that under unilateral ANT stimulation, epileptic rats have an approximately 50% reduction in total seizure number compared with the KA group. This is in accordance with previous studies. Epileptogenesis is a process by which a normal brain is transformed into an epileptic one. After the administration of KA, rats will experience a process of epileptogenesis and, eventually undergo spontaneous seizures. At present, many strategies have been pursued to inhibit this epileptogenesis process and prevent the emergence of epilepsy. These include antiepileptic drugs such as valproate, gabapentin, and diazepam, nonantiepileptic drugs such as rapamycin, losartan, and anti-inflammatory drugs. Nevertheless, many of these treatments are unsatisfactory. In our study, we found that the latency in ANT stimulation group is longer, which implicates that ANT stimulation not only could inhibit the hyperexcitability in epilepsy but also modulate the epileptogenesis process.

In the hippocampus, the progressive loss of neurons occurred during the process of epileptogenesis. Previous research...
has revealed that neurons in the CA1 and CA3 regions of the hippocampus were more vulnerable to injuries in the KA-induced epileptic animal models.\[25‑27\] Our research has shown significant neuronal loss in the CA3 region of the hippocampus in the KA and sham-DBS groups. With ANT stimulation, neuronal loss is attenuated. This demonstrated that chronic ANT stimulation could protect against neuronal loss in the hippocampus, which might be one of the underlying mechanisms of ANT-DBS in the treatment of epilepsy.

Neuronal apoptosis is common in several neurodegenerative diseases. It has been demonstrated that neuronal apoptosis contributes to neuronal loss in the sclerotic hippocampus.\[5‑7\] During apoptosis, CASP3 is a key mediator. The apoptotic signals from the mitochondrion-mediated pathway and Fas-mediated pathway activate CASP3 and induce apoptosis. The Bcl2 family could regulate cell apoptosis by regulating the mitochondrion-mediated apoptosis pathway. BCL2 is located on the membrane of mitochondria and the endoplasmic reticulum. The activation of BCL2 can inhibit apoptosis by stabilizing the membrane of mitochondria, thereby preventing the release of glutathione and cytochrome c.\[26\] BAX is a pro-apoptotic protein that is mainly located in the plasma. Once activated, it combines with BCL2 and inhibits its anti-apoptotic function.\[29\] We evaluated the protein and mRNA levels of Casp3, Bcl2, and Bax in the hippocampus. In epileptic rats, the expression of Casp3 and Bax increased, and the expression of Bcl2 decreased, but after chronic ANT stimulation, some of these changes returned to the control level. Therefore, this finding indicates that chronic ANT stimulation could exert anti-apoptotic effects on hippocampal neurons. This effect may contribute to the reduced neuronal loss in the epileptic hippocampus. Interestingly, after stimulation, the levels of Bax mRNA remained higher compared with KA group whereas the BAX protein levels decreased in the DBS group. This may indicate the transcriptional regulation effect of ANT-DBS on Bax.

Although the anti-apoptotic effect of ANT-DBS has been demonstrated in our research, the concrete mechanism is not fully understood. Previous research has found that ANT-DBS could balance the neurotransmitter levels and ameliorate glucose metabolism in the epileptic hippocampus.\[15,30\] After ANT stimulation, the extracellular concentration of GABA increases whereas the extracellular concentration of glutamate decreases. The increase of GABA will increase the activity of GABA receptor. It has been demonstrated that the co-activation of inotropic and metabotropic GABA receptor protected against neuronal apoptosis induced by KA.\[31\] Therefore, this inhibition effect will reduce the apoptosis and neuronal loss. Adenosine, which has been demonstrated to have antiepileptic and neuroprotective effects, was also observed to be elevated in the hippocampus after ANT stimulation.\[32\] It has been determined that adenosine could protect neurons against apoptosis through activating adenosine A1 receptors. This function is conducted by the regulation of the mitogen-activated protein kinase and serine/threonine kinase pathways, which have been demonstrated to regulate cell differentiation, survival, and apoptosis.\[33,34\] Whether the anti-apoptotic effect of ANT stimulation may also be mediated by these mechanisms or other mechanism deserves further evaluation.

In conclusion, increases in apoptosis and marked neuronal losses in the hippocampus of KA-induced epileptic rats were found in our study. Chronic unilateral ANT stimulation can significantly reduce seizure activity and attenuate both apoptosis and neuronal loss in the epileptic hippocampus. These findings indicate that ANT stimulation could exert protective effects on hippocampal neurons and further our understanding of the mechanism of ANT stimulation in the treatment of epilepsy.

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Conflicts of interest
There are no conflicts of interest.

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