Selective Upregulation of Brain-derived Neurotrophic Factor (BDNF) Transcripts and BDNF Direct Induction of Activity Independent N-Methyl-D-aspartate Currents in Temporal Lobe Epilepsy Patients with Hippocampal Sclerosis

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Brain-derived neurotrophic factor (BDNF) plays a critical role in many aspects of neuronal biology and hippocampal physiology and pathology, and has been implicated as a potential therapeutic target in temporal lobe epilepsy (TLE). BDNF total mRNA and its six transcripts were compared in the hippocampal tissue of TLE patients with or without hippocampal sclerosis (HS) by real-time fluorescence quantitative polymerase chain reaction. Excitatory actions induced by BDNF on hippocampal cells were investigated by whole-cell patch-clamp recordings. Statistically significant increases in three human BDNF mRNA transcripts were observed in TLE patients with HS compared with those without HS (transcripts 2, 3 and 5 exhibited 2.1-, 2.3- and 4.1-fold increases, respectively); there were no significant increases in other transcripts. BDNF directly induced N-methyl-D-aspartate currents in dentate granule cells of TLE patients with HS. These results demonstrated that BDNF transcripts were selectively upregulated in TLE patients with HS compared with those without HS. Moreover, BDNF induced excitability of dentate granule cells in TLE patients with HS.

KEY WORDS: TEMPORAL LOBE EPILEPSY; HIPPOCAMPAL SCLEROSIS; BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) mRNA TRANSCRIPTS; N-METHYL-D-ASPARTATE (NMDA); REAL-TIME FLUORESCENCE QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (REAL-TIME qRT–PCR)

Introduction

Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures. According to the World Health Organization,1 around 50 million people worldwide have epilepsy. This disease is usually controlled by medication but, unfortunately, cannot be cured. There are
more than 40 different types of epilepsy. Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults who experience seizures that are poorly controlled with anticonvulsant medications and, in most cases, the epileptogenic region is found in the midline temporal structures such as the hippocampus. The hippocampus is a major part of the human brain and plays important roles in long-term memory and spatial navigation. The hippocampus has four subdivisions: CA1 to CA4. The dentate gyrus is the main hippocampal input structure, receiving strong excitatory cortical afferents via the perforant path. Repetitive perforant-path stimulation at 5 – 10 Hz can induce epileptiform bursts in minislices of dentate gyrus from rats with kainate-induced epilepsy. Morphological investigations have revealed that TLE is associated with hippocampal sclerosis (HS), characterized by partial or complete loss of neuronal cells and aberrant mossy fibre sprouting, especially in the CA1 and CA3 areas of the hippocampus, while cell loss is seldom associated with non-HS. Aberrant mossy fibre sprouting is thought to be involved in increased recurrent activation of granule cells in TLE.

Brain-derived neurotrophic factor (BDNF) is a member of the neutrophin family of growth factors. It is involved in numerous aspects of development, degeneration and differentiation in the central nervous system and appears to play an important role in epileptogenesis in the hippocampus. Following epileptogenic stimuli, dendritic targeting of BDNF mRNA that may contribute to the cellular changes leading to epilepsy was observed in the rat hippocampus. The human BDNF gene has multiple promoters governing six non-coding upstream exons that are spliced to one downstream coding exon, resulting in six different transcripts. This complicated transcriptional regulation leads to at least three pre-pro-BDNF isoforms. Pro-BDNF is an apoptotic ligand that can induce death at subnanomolar concentrations. BDNF potentiates excitatory synapses through its high-affinity receptor tyrosine protein kinase B (TrkB). Inhibition of this receptor can significantly reduce aberrant mossy fibre sprouting and modify epileptic activity in a model of TLE. Upregulated BDNF was confirmed in a number of chronic seizure models in animals and in human epileptic brains.

N-Methyl-D-aspartic acid (NMDA) is an amino acid derivative that acts as a specific agonist at the NMDA receptor, mimicking the action of glutamate, which normally binds to that receptor. Activation of the NMDA receptor results in the opening of an ion channel that is non-selective to cations and plays an important role in cell death during status epilepticus. In patients with TLE, the levels of NR2A and NR2B (two isoforms of the heterometric NMDA receptor subunit 2) per dentate granule cell have been found to increase significantly, compared with autopsy hippocampi. BDNF can regulate synaptic transmission by increasing NMDA receptor activity, reduce inhibition on GABAergic postsynaptic cells at GABAergic synapses in hippocampal cultures, evoke rapid excitation on neurons in rat hippocampi rats, and promote epileptogenesis by TrkB signalling in a mouse model of mesial temporal lobe epilepsy.

The present study was designed to investigate the changes of BDNF mRNA transcripts in the hippocampus of patients with TLE, with or without HS, and examined the effect of BDNF on dentate granule cells in patients with TLE and HS by whole-cell patch clamp recording in brain slices.
Patients and methods

PATIENT SELECTION AND TISSUE COLLECTION

Surgically removed tissue specimens (0.5 cm in size) from the hippocampus were collected from consecutive patients with intractable TLE who were operated on in the First Affiliated Hospital of Harbin Medical University, Harbin, China. Specimens were collected between April 2008 and April 2010. All patients with TLE were confirmed and HS was defined according to previously published standards.29 Patients with bilateral HS or dual pathology (HS accompanied by other epileptogenic lesion) were excluded. After en bloc resection, the hippocampus was cut into three slices perpendicular to its long axis and the middle portion was used for analysis. The HS specimens from the same tissues that were to be evaluated by patch-clamp recording were placed in an oxygenated chamber with sucrose-based artificial cerebrospinal fluid (SACSF) for brain slice preparation. Residual tissue specimens were frozen in liquid nitrogen and processed for RNA extraction.

This project was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. All patients gave written informed consent for the research use of their biopsy materials.

PREPARATION OF HUMAN HIPPOCAMPAL SLICES

Surgically resected hippocampal specimens were collected directly from the patients by neurosurgeons from the First Affiliated Hospital of Harbin Medical University and immediately immersed in oxygenated ice-cold SACSF containing 213 mM sucrose, 2.5 mM potassium chloride, 1.25 mM sodium phosphate monobasic monohydrate, 10 mM dextrose, 2 mM magnesium sulphate and 2 mM calcium chloride. In < 3 min, the specimens were transferred to the laboratory. Transverse hippocampal slices of 300 – 400 µm thick were prepared using a vibratome (NVSLM1; World Precision Instruments, Sarasota, FL, USA) and then incubated at room temperature (22 – 25 °C) in oxygenated (5% carbon dioxide, 95% oxygen) artificial cerebrospinal fluid (ACSF) containing 126 mM saline, 2.5 mM potassium chloride, 1.25 mM sodium phosphate monobasic monohydrate, 26 mM sodium bicarbonate, 10 mM dextrose, 2 mM magnesium sulphate and 2 mM calcium chloride.

REAL-TIME qRT–PCR ANALYSIS OF BDNF mRNA TRANSCRIPTS

Total cellular RNA was purified from hippocampal samples using TRIzol® reagent (TransGen Biotech Co. Ltd, Beijing, China) following the manufacturer’s protocol, and was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Shiga, Japan). Assays were performed using a Mx3000™ real-time PCR system (Stratagene, La Jolla, CA, USA). The primers and probes (Table 1) used in the real-time fluorescence quantitative reverse transcription–polymerase chain reaction (real-time qRT–PCR) system were designed and synthesized by Shanghai Chaoshi Biotechnology (Shanghai, China). Assays were performed using a Mx3000™ real-time PCR system (Stratagene, La Jolla, CA, USA). The initial amplification programme consisted of an activation step (50 °C for 2 min, then 95 °C for 10 min), followed by 40 cycles of denaturation (95 °C, 30 s) and annealing and elongation (60 °C, 30 s). The results are presented as crossing threshold (Cₜ), which is defined as the PCR cycle at which sample fluorescence crosses a threshold set above baseline fluctuations and background noise, and is within the logarithmic portion of the amplification plot. The difference in crossing thresholds (ΔCₜ) was calculated for each cDNA being studied:
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\[ \Delta C_T = C_T (\text{BDNF transcripts of interest}) - C_T (\beta\text{-actin}) \]

The mean ± SD was determined for each group. The differences between groups were calculated as \( \Delta \Delta C_T = \Delta C_T \) (HS group) – \( \Delta C_T \) (non-HS group). Relative mRNA expression was estimated by \( 2^{-\Delta \Delta C_T} \). 30

### REAGENTS FOR WHOLE-CELL PATCH-CLAMP RECORDING

Recombinant human BDNF, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), biocytin, DL-2-amino-5-phosphonopentanoic acid (APV), bicineuline methiodide and lidocaine N-ethyl bromide (QX-314) were purchased from Sigma-Aldrich (St Louis, MI, USA). BDNF was dissolved in sterile water to make a stock solution of 10 mg/ml. Before each experiment, the recording chamber and tubing were flushed with ACSF and 0.005% bovine serum albumin (BSA) for 10 min. BSA was added to ACSF at a concentration of 0.001% for both the pre- and post-treatment recordings. CNQX was initially dissolved in dimethyl sulfoxide and applied to the slices with a final concentration of 20 μM.

### WHOLE-CELL PATCH-CLAMP RECORDING

Granule cells in dentate gyrus were identified using infrared differential interference contrast videomicroscopy with a fixed-stage microscope, fitted with a 40x/0.80W lens (Nikon F-S adaptor; Nikon Corp. Instruments Co., Sendai, Japan). Patch electrodes with a resistance of 3 – 5 MΩ were filled with bath solution containing 140 mM potassium gluconate, 10 mM 4-(2-hydroxyethyl)-1-

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**TABLE 1:**
The sequences of primers and probes for brain-derived neurotrophic factor (BDNF) transcripts

<table>
<thead>
<tr>
<th>BDNF transcript</th>
<th>Primers</th>
<th>Product</th>
</tr>
</thead>
</table>
| Transcript 1    | Forward: 5′-TTACAATCAGATGGGCCACATG-3′  
Re reverse: 5′-GGAGAAAGCAGAAACACAGACAGAAA-3′  
Probe: FAM-ATCCCCGTGAAAGAAAGCCCTAACCAG-TAMRA | 77 bp |
| Transcript 2    | Forward: 5′-GGAGCGGGGCTTGGGA-3′  
Re reverse: 5′-CCAACTTCAGCAGCTCAATT-3′  
Probe: FAM-AGCGAGCCCGCTTGGTCCCCC-TAMRA | 62 bp |
| Transcript 3    | Forward: 5′-GCAAAGGCGAACTTCACC-3′  
Re reverse: 5′-TTAATCTCGTCCTCCCCAAGAC-3′  
Probe: FAM-ATGACCTCAAACAAGACACATTCCCTCAGCA-TARMA | 79 bp |
| Transcript 4    | Forward: 5′-GGGACCCGCTTGAGTTTG-3′  
Re reverse: 5′-ACCATAAGGAAAGGATGTCTAAT-3′  
Probe: FAM-ACCCGAGCTCACCAGGTAAGAAG-TAMRA | 77 bp |
| Transcript 5    | Forward: 5′-CATACCGGCGCCACAGACT-3′  
Re reverse: 5′-TTTCAGCTCCTCCTCCGCTA-3′  
Probe: FAM-CGCCGCTCCTCCTCCTTAA-TARMA | 63 bp |
| Transcript 6    | Forward: 5′-AACGTAAAGGGTGCTCAGAC-3′  
Re reverse: 5′-GCCGAGATGGGTAGAGA-3′  
Probe: FAM-TCCGAGAGCCGCAACCA-TAMRA | 62 bp |

bp, base pairs.  
FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.
TABLE 2: Demographic and clinical characteristics of patients with temporal lobe epilepsy, with or without hippocampal sclerosis (HS)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HS group (n = 12)</th>
<th>Non-HS group (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, males/females</td>
<td>7/5</td>
<td>6/4</td>
</tr>
<tr>
<td>Age, years</td>
<td>25.91 ± 5.51</td>
<td>22.40 ± 7.97</td>
</tr>
<tr>
<td>Epilepsy duration, years</td>
<td>13.33 ± 5.43</td>
<td>14.9 ± 5.33</td>
</tr>
<tr>
<td>Site of resection, left/right</td>
<td>8/4</td>
<td>7/3</td>
</tr>
</tbody>
</table>

Data presented as mean ± SE or number of patients. Between-group differences were not statistically significant (P > 0.05); χ²-test for sex and site of resection, or one-way analysis of variance test for age and epilepsy duration.

piperazine-ethanesulphonic acid, 3 mM potassium chloride, 4 mM magnesium chloride, 0.2 mM ethylene glycol tetra-acetic acid, 4 mM disodium adenosine 5′-triphosphate, 0.5% biocytin and 5 mM QX-314; the pH was 7.3. All recordings were made at room temperature (22 – 25°C). Holding potentials were stepped from −80 mV to +80 mV with an increment of 20 mV (Axopatch™ 200B, signal 3.06; Axon Instruments Inc., Union City, CA, USA). BDNF was diluted with ACSF to a final concentration of 100 ng/ml and applied using a Picospritzer III (Parker Hannifin Corp., Fairfield, NJ, USA) linked to micropipettes. The pipettes were placed at a distance of approximately 15 µm above the soma. Access resistance (10 – 25 MΩ) was regularly monitored during recordings and the data were rejected if the fluctuation was > 15%. Heat-inactivated BDNF was used for the controls.

STATISTICAL ANALYSES
Data were analysed using the SAS software package, version 8.2 (SAS Institute Inc., Cary, NC, USA). Quantitative data are presented as means ± SD. Statistical analyses were performed using analysis of variance followed by a paired-samples t-test and χ²-test for comparison between groups. A P-value < 0.05 was considered to be statistically significant.

Results
The demographic and clinical characteristics of patients with and without HS are summarized in Table 2. The inclusion criteria were met by 22 patients (12 patients with HS and 10 patients without HS). Patient’s ages at surgery ranged from 10 to 35 years. There were no statistically significant differences in patients with or without HS with respect to sex, age, epilepsy duration or side of resection (right or left).

A significant increase in BDNF mRNA was observed in patients with TLE and HS, compared with those without HS (P < 0.01; Fig. 1). Statistically significant increases in levels of three of the human BDNF mRNA transcripts were observed in TLE patients with HS, compared with those without HS: transcripts 2, 3 and 5 exhibited 2.1-, 2.3- and 4.1-fold increases, respectively (P < 0.01; Fig. 2). There were no significant increases in the other transcripts.

Experiments were conducted on neurons located in the hippocampal granule cell areas of patients with HS; results for 12 neurons are given in Fig. 3. Excitatory postsynaptic current (EPSC) rose rapidly when BDNF (100 ng/ml) was applied locally.
(approximately 15 \text{ \mu m} above the soma) in the presence of the non-NMDA receptor antagonist CNQX (20 \text{ \mu M}) and the GABA_A receptor antagonist bicuculline methiodide (25 \text{ \mu M}) (Fig. 3A). The mean ± SD latency of the BDNF-evoked depolarization was 178 ± 7.8 ms. The voltage dependence of BDNF-evoked currents that were measured at holding potentials from –80 mV to +80 mV were determined and it was found that the mean ± SD experimental reversal potential was approximately 12.7 ± 5.4 mV. The EPSCs exhibited a non-linear current–voltage relationship and displayed a peak inward current at –20 mV (Fig. 3B). It was then observed that application of heat-inactivated BDNF was entirely ineffective in four out of four neurons. After application of APV (50 \text{ \mu M}), a selective NMDA receptor antagonist that competitively inhibits the ligand-binding site of the NMDA receptor, the currents were completely blocked within 5 min (Fig. 3A). These results clearly indicated that BDNF directly evoked NMDA currents. The morphology of granule cells filled with biocytin during the patch-clamp experiments was also evaluated (Fig. 3C).

**Discussion**

Seizure activity increased both mRNA and protein levels of BDNF in the rodent hippocampus,\textsuperscript{31} – \textsuperscript{33} and spontaneous seizures were reduced by local delivery of BDNF in an epilepsy model.\textsuperscript{34} In a pilocarpine-induced seizure model, transcript 2 made the greatest contribution to pilocarpine effects on total BDNF mRNA content and the Ca\textsuperscript{2+} channel-directed selective expression of the BDNF transcript 2.\textsuperscript{35} Calcium influx activated the cAMP
Six transcripts have been reported for the human BDNF gene; however, expression of all six transcripts has not been confirmed in patients with TLE. The present study demonstrated that not only are all six transcripts present in patients with TLE, but that a significant increase in transcripts 2, 3 and 5 can be detected in those who also have HS, compared with those who only have TLE.

A large body of evidence implicates the interplay of the NMDA receptor and BDNF in patients with TLE. A putative cellular basis for recurrent epileptic seizures is that aberrant sprouting and synaptic reorganization of the mossy fibre axons might cause excitatory feedback loops in the dentate gyrus. Dentate gyrus and hilus transection blocked seizure propagation and granule cell dispersion in a mouse model of mesial temporal lobe epilepsy. Also, BDNF may have established hyperexcitable dentate circuits by induced axonal branching. The activity of NMDA receptors could increase BDNF mRNA levels through the calcium signalling pathway, mediate BDNF exon IV transcription in cultured cortical neurons by phosphoinositide 3-

**FIGURE 2:** Levels of brain-derived neurotrophic factor (BDNF) mRNA in transcripts 1 – 6 in patients with temporal lobe epilepsy, with or without hippocampal sclerosis (HS), as measured by real-time fluorescence quantitative reverse transcription–polymerase chain reaction. The mean crossing threshold ($\Delta C_T$) was calculated for each isoform: $\Delta C_T = C_T$ (BDNF mRNA transcripts of interest) – $C_T$ (\(\beta\)-actin). Relative expression between groups was determined by $2^{-\Delta \Delta C_T}$ where $\Delta \Delta C_T = \Delta C_T$ (HS) – $\Delta C_T$ (non-HS) (data normalized to \(\beta\)-actin; data are presented as mean ± SD; each experiment was repeated twice for each probe and each sample was run three times; **$P < 0.01$ versus without HS)
kinase and phospholipase C signalling pathways and stimulate postsynaptic BDNF secretion through calcium influx. BDNF has been found to increase the NMDA receptor-gated Ca\(^{2+}\) influx into dendrites of neurons with Ca\(^{2+}\) oscillations by confocal laser microscopy in rats. Activation of TrkB receptors of BDNF potentiated NMDA

**FIGURE 3:** Brain-derived neurotrophic factor (BDNF) (100 ng/ml) evokes N-methyl-D-aspartate (NMDA) excitatory postsynaptic currents (EPSC). (A) Example of EPSC time courses in a dentate granule cell evoked by puffing BDNF (100 ng/ml): holding potential indicated on the right of each current trace. Peak current occurred at 178 ± 7.8 ms postpuffing (n = 12 neurons). Each current trace is the mean of four to eight individual traces. Upper current trace illustrates that NMDA-EPSCs were blocked by bath application of the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (APV) (50 µM, 5 min). The lower series of current traces show EPSCs measured at nine different holding potentials. (B) Relationship between holding potential and peak NMDA-EPSC current in neurons from Fig. 3A. Data collected from 12 neurons in granule cells represented as mean ± SD. Like all NMDA currents, the current–voltage relationship presented here is not linear, with significant rectification at the hyperpolarized voltage. Peak inward current occurs at –20 mV. (C) Representative biocytin-filled granule cells in dentate gyrus of temporal lobe epilepsy patients with hippocampal sclerosis.
receptor currents in *Xenopus* oocytes microtransplanted with rat forebrain postsynaptic densities. Moreover, BDNF has been found to increase NMDA receptor single channel open probability, resulting in enhancement of synaptic transmission in cultures of hippocampi.

Brain-derived neurotrophic factor has been implicated in sprouting in TLE, and the resulting increase in NMDA glutamate receptors appears to mediate increased recurrent activation. The NMDA-Ca transduction pathway leads to neuronal death in a time-dependent manner with *in vitro* status epilepticus. It has been suggested that BDNF increases the amplitude and frequency of spontaneous excitatory postsynaptic currents. BDNF modulated glutamate release from mossy fibre terminals by a presynaptic action in area CA3. Acute intrahippocampal infusion of function-blocking antibodies against BDNF prevented neuronal degeneration after status epilepticus. The electrophysiological findings from the present study suggest, however, that BDNF itself can directly elicit NMDA-EPSC in hippocampal dentate granule cells, even without glutamate. Although the underlying mechanism has yet to be clarified, this novel effect of BDNF might exert an additional background excitation on the granule cells in an activity independent manner, resulting in more deleterious excitatory toxicity in TLE patients with HS.

In conclusion, the present study demonstrated that three of the six human BDNF mRNA transcripts are upregulated and that BDNF can directly induce activity independent NMDA receptor-mediated currents in TLE patients with HS, which might be the cause of overexcitation in these patients. The study also suggests that BDNF may be a potential target for new antiepileptic drugs.

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**Conflicts of interest**

The authors had no conflicts of interest to declare in relation to this article.

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