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2-1-2015

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#### Recommended Citation

Semaan, Suzan; Wu, Jie; Gan, Yan; Jin, Yu; Li, Guo Hui; Kerrigan, John F.; Chang, Yong; and Huang, Yao, "Hyperactivation Of Bdnf-Trkb Signaling Cascades In Human Hypothalamic Hamartoma (Hh): A Potential Mechanism Contributing To Epileptogenesis" (2015). *Translational Neuroscience*. 55.

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# Hyperactivation of BDNF-TrkB Signaling Cascades in Human Hypothalamic Hamartoma (HH): A Potential Mechanism Contributing to Epileptogenesis

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## Keywords

Brain-derived neurotrophic factor; Epileptogenesis; Human hypothalamic hamartoma; Signal transduction; Tropomyosin-related kinase B.

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Received 20 June 2014; revision 20 August

2014; accepted 4 September 2014

doi: 10.1111/cns.12331

## Introduction

Human hypothalamic hamartoma (HH) is a developmental malformation that occurs in the region of the tuber cinereum and inferior hypothalamus. HH is associated with diverse neurological and endocrine disorders, including epilepsy, cognitive impairment, behavioral disturbances, and central precocious puberty [1,2]. The epileptic syndrome in HH patients is often characterized by gelastic (laughing) seizures beginning in early infancy, followed by the development of additional seizure types, which are usually refractory to available anti-epilepsy drugs (AEDs) and alternative therapies [2]. HH is a human model of subcortical epilepsy [3]. There is now compelling evidence that HH is intrinsically epileptogenic for gelastic seizures, including seizure recordings with surgically implanted electrodes and positive results for seizure control with surgical resection [4,5]. However, the molecular and cellular mechanisms responsible for epileptogenesis within HH are largely unknown.

Electrophysiological features of HH neurons suggest that gamma-aminobutyric acid (GABA)-mediated excitation plays an

## SUMMARY

**Aims:** Although compelling evidence suggests that human hypothalamic hamartoma (HH) is intrinsically epileptogenic for gelastic seizures, the molecular mechanisms responsible for epileptogenesis within HH remain to be elucidated. The aim of this study was to test the hypothesis that hyperactivation of BDNF-TrkB signaling pathways in surgically resected HH tissue is a possible mechanism for downregulation of KCC2 expression, which in turn underlies GABA-mediated excitation within HH. **Methods:** Activation of three major BDNF-TrkB signaling pathways including MAPKs, Akt, and PLC $\gamma$ 1 were evaluated in surgically resected HH tissue (n = 14) versus human hypothalamic control tissue (n = 8) using combined methodologies of biochemistry, molecular biology, cell biology, and electrophysiology. **Results:** Our data show that compared with hypothalamic control tissue, in HH tissue, (i) activation of TrkB and expression of mature BDNF are elevated; (ii) MAPKs (including ERK1/2, p38, and JNK), Akt, and PLC $\gamma$ 1 are highly activated; (iii) KCC2 expression is downregulated; and (iv) pharmacological manipulation of TrkB signaling alters HH neuronal firing rate. **Conclusion:** Our findings suggest that multiple BDNF-TrkB signaling pathways are activated in HH. They act independently or collaboratively to downregulate KCC2 expression, which is the key component for GABA-mediated excitation associated with gelastic seizures.

important role in epileptogenesis [6–8]. Binding of GABA to its ionotropic GABA<sub>A</sub> receptor, a ligand-gated Cl<sup>-</sup> channel, usually leads to the hyperpolarization of neuronal membranes via the influx of Cl<sup>-</sup> ions [9]. However, GABA can exert an excitatory role in immature brain and in mature brain under certain pathophysiological conditions including epilepsy [10]. The key mechanism underlying GABA-induced neuronal excitation relates to the intracellular Cl<sup>-</sup> concentration and the transmembrane Cl<sup>-</sup> gradient. Maintenance of the Cl<sup>-</sup> gradient required for hyperpolarizing ionotropic responses (relatively low intracellular Cl<sup>-</sup> concentration) is attributable to the functional expression of the mature neuron-specific K<sup>+</sup>-Cl<sup>-</sup> cotransporter, KCC2 [11]. We have observed that most large HH neurons, which appear to be excitatory projection-type pyramidal neurons, demonstrate depolarization and increased firing in response to GABA, in association with relatively low levels of KCC2 expression and elevated intracellular Cl<sup>-</sup> concentrations [6,7,12,13]. As a result, GABA exerts an excitatory role in these neurons, potentially contributing to seizure genesis. Here, we hypothesized that downregulation of KCC2 expression related to alterations in key intracellular signal

transduction pathways in HH tissue that have not been previously investigated.

Brain-derived neurotrophic factor (BDNF), a member of the family of neurotrophins, is essential for neuronal development and is implicated in the modulation of synaptic function and plasticity [14,15]. BDNF exerts its biological effects via its high-affinity receptor, TrkB, a member of the tropomyosin-related kinase (Trk) family of the receptor tyrosine kinase (RTK) superfamily. BDNF binding leads to TrkB dimerization and intrinsic kinase activation. This results in trans-autophosphorylation on multiple tyrosine residues within the intracellular domain of TrkB, which potentiates the receptor tyrosine kinase activity and creates docking sites for adaptor proteins, such as Shc and phospholipase C $\gamma$  (PLC $\gamma$ ). These adaptor proteins couple the receptor to several important intracellular signaling cascades, including the Ras-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-Akt, and PLC $\gamma$ -Ca<sup>2+</sup> pathways [16,17]. These pathways regulate gene expression and protein translation [18].

It has been previously reported that TrkB activation can reduce GABA<sub>A</sub> receptor-mediated responses of CA1 pyramidal cells [19,20]. One possible mechanism by which TrkB impairs GABAergic inhibition is through downregulation of KCC2 expression. This is supported by the following findings: (i) BDNF-mediated TrkB activation resulted in reduced KCC2 expression and impaired neuronal Cl<sup>-</sup> extrusion in rat hippocampal slice cultures; (ii) in kindled mice, KCC2 expression was downregulated in hippocampus with a spatio-temporal profile complementary to the upregulation of BDNF and TrkB; (iii) hippocampal slices derived from transgenic *trkB* mutant mice (*trkB*<sup>SHC/SHC</sup> and *trkB*<sup>PLC/PLC</sup>) suggested that activation of both Shc and PLC $\gamma$  signaling cascades is required for TrkB-mediated downregulation of KCC2 [11,21,22].

In this study, we aimed to test the hypothesis that hyperactivation of BDNF-TrkB signaling pathways in surgically resected HH tissue is a possible mechanism for downregulation of KCC2 expression, which in turn underlies GABA-mediated excitation within HH.

## Materials and Methods

### Clinical Profiles of Study Subjects

The use of surgically resected tissue in this study was approved by the Institutional Research Board (IRB) of St. Joseph's Hospital and Medical Center. HH tissue was extracted from 14 patients (mean age 8.5 years, range 0.7 to 40.8 years; 7 [50%] were female). All patients had treatment-resistant epilepsy and had failed at least three AEDs. The mean age for seizure onset was 5.6 months (range 1 to 18 months; 7 [50%] had first seizure activity before one month of age). At the time of surgery, 6 (43%) had only gelastic seizures, while 8 (57%) had multiple seizure types. All patients had gelastic seizures at some point during their clinical course. Twelve patients (86%) experienced at least one seizure every day, while two patients (14%) had at least one seizure every month. At surgery, nine patients (64%) were taking at least 2 AEDs (range 0 to 4 AEDs). Prior treatment for epilepsy had included gamma knife radiosurgery in three patients (21%). Seven patients (50%) had a history of intellectual disability (intelligence quotient [IQ] or developmental quotient [DQ] <50). Four

patients (29%) had a history of central precocious puberty. None of the patients in this cohort had Pallister-Hall syndrome or other identified syndromes. The mean HH lesion volume was 3.2 cm<sup>3</sup> (range 0.2 to 20 cm<sup>3</sup>). All HH tissue used in Western blot analysis was snap frozen on dry ice immediately after surgical resection and stored at -80°C until assay.

### Human Control Tissue

Postmortem (frozen) hypothalamic tissue derived from 8 normal human donors (two females and six males) was obtained from the Harvard Brain Tissue Resource Center, the Human Brain & Spinal Fluid Resource Center (West Los Angeles Healthcare Center), and the University of Maryland. Brain tissue was collected within 4 h after death. The mean age was 39 years, range 12.7–78 years.

### Antibodies and Reagents

Primary antibodies used are detailed in Table S1. The Trk kinase inhibitor K252a was from EMD Millipore.

### RNA Extraction and Real-Time RT-PCR

Total RNAs were extracted from tissue using QIAzol Lysis Reagent (QIAGEN, Valencia, CA, USA). cDNA synthesis and quantitative PCR were performed as previously described [6,7,23].

### Protein Extraction, SDS-PAGE, and Immunoblotting

Tissue samples (~50 mg) were dissected from frozen sections, weighed, thawed on ice, and lysed in ice-cold RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors [24]. Following homogenization with plastic pestles that tightly fit microtubes, the homogenates were sheared 5 times by passing through single-use needles (0.4 × 13 mm) (Becton Dickinson, Franklin Lakes, NJ, USA) and solubilized for 1 h on ice. After centrifugation, the supernatants (protein extracts) were collected, quantitated and resolved on SDS-PAGE [23,24]. Immunoblotting with antibodies (Table S1) was performed using standard procedures. Immunoblotting signals were detected with SuperSignal chemiluminescent substrate (Pierce) and images were captured using a Kodak 4000 MM molecular imager [23,25,26].

### Immunohistochemical Staining

Tissue sections were prepared and immunostaining with a goat anti-KCC2 antibody (R-14) (Santa Cruz Biotechnology) was performed as described previously [6,8].

### Patch-Clamp Recordings in HH Slices

Acute HH slices were prepared as previously described [6–8]. The conventional whole-cell recordings were made using a patch-clamp amplifier (Multiclamp 700A, Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) under infrared-DIC (differential

interference contrast) microscopy. The patch-pipette solution contained (in mM): 140 K<sup>+</sup>-gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl<sub>2</sub>, 4 MgATP, 0.3 Na<sub>2</sub>GTP and 10 Na<sub>2</sub>-phosphocreatine (pH7.3 with KOH). HH neuron action potential was recorded in current-clamp mode. Whole-cell access resistance <20 MΩ was accepted for experiments. The series resistance was not compensated in this study. Typically, data were acquired at 10 kHz, filtered at 2 kHz, displayed and digitized online (Digidata 1322 series A/D board, Axon Instruments), and stored to a hard drive. Data acquisition and analyses of action potential firing using patch-clamp whole-cell recording in current-clamp mode were done using Clampex 9.2 and Clampfit 9.2 (Axon Instruments), respectively, and results were plotted using Origin 5.0 (Microcal, OriginLab, Northampton, MA, USA). The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose and 10 HEPES (pH adjusted to 7.4 with Tris-base). All experiments were performed at room temperature (22 ± 1°C).

## Densitometry and Statistical Analysis

Densitometric quantification of digital immunoblotting images was performed using Kodak Molecular Imaging Software (Version 4.0). Data were given as mean ± SEM. The significance (*P* value) was calculated with analysis of variance (ANOVA) or paired or unpaired *t*-tests when applicable. Pearson correlation analysis was performed using GraphPad Prism (Version 5.0) to evaluate the relationships among the variables of interest. *P* < 0.05 was considered statistically significant.

## Results

### Enhanced Overall Tyrosine Phosphorylation Signals in HH Tissue

Tyrosine phosphorylation is one of the key steps in intracellular signal transduction and regulation of enzymatic activity in eukaryotic cells, and perturbations in tyrosine phosphorylation

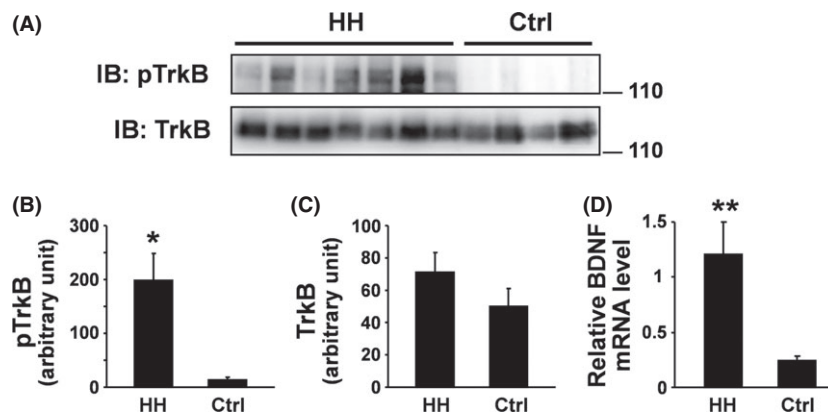
underlie many human diseases [27,28]. We sought to assess the overall tyrosine phosphorylation levels in both HH tissue and normal human hypothalamus (control) by immunoblotting analysis.

Proteins extracted from HH (*n* = 14) and control (*n* = 8) tissue were resolved on SDS-PAGE and further analyzed by immunoblotting with a mouse monoclonal anti-phosphotyrosine antibody (pTyr, 4G10). This antibody is very sensitive and reliable for detection of tyrosine phosphorylation and has been successfully used in our previous studies [24–26]. Our results indicated that the overall phosphorylation level in HH tissue was markedly higher than in control tissue (Fig. S1).

This experimental approach utilizes quantitative assessment of phosphorylated protein levels relative to total protein levels by densitometric analysis of immunoblotting results. This technique has been successfully employed in our previous studies [23–26]. However, the impact of postmortem interval (PMI) on protein phosphorylation and total protein levels could be a concern given the nature of tissue collection. To address this, we performed a set of immunoblotting experiments using rat brain tissues collected 1–4 h after death of the animals at room temperature (22 ± 1°C). Representative images of immunoblotting with anti-active-ERK1/2 (pERK1/2) and anti-total ERK1/2 blots are shown in Fig. S2. Both antibodies have been well documented in our previous studies [23,24,26]. Our results consistently demonstrated that PMI had very little effect on both protein phosphorylation and total protein levels at least within the initial 4 h after death (Fig. S2). This ensures the accuracy of our immunoblotting data throughout the study.

### Elevated Phosphorylation of TrkB and Expression of BDNF in HH Tissue

Increased overall phosphorylation in HH versus control tissue prompted us to further examine BDNF-TrkB signaling. We first assessed TrkB phosphorylation by immunoblotting with an anti-phospho-TrkB (Tyr816) (pTrkB) antibody, and detected enhanced immunoreactive signals in HH versus control group (Figure 1A).



**Figure 1** Enhanced BDNF-TrkB signaling in HH. (A–D) Elevated phosphorylation of TrkB in HH. Total proteins were extracted from HH and normal human hypothalamic control tissue specimens and equal amounts of proteins were subjected to SDS-PAGE and analyzed by immunoblotting with anti-phospho-TrkB (Tyr816) (pTrkB) and anti-TrkB, respectively (A). Densitometric analysis results of immunoblotting data of anti-pTrkB (B) and anti-TrkB (C), respectively, from 14 HH and 8 controls are shown. (D) Elevated BDNF expression (mRNA) in HH. Total RNAs were extracted from HH (*n* = 16) and control (*n* = 3) tissue specimens and used for real-time RT-PCR analysis to measure the mRNA level of mature BDNF relative to that of human GAPDH. \**P* < 0.05; \*\**P* < 0.01.

In contrast, the expression of TrkB was detected by anti-TrkB antibody in all samples (Figure 1A). The levels of pTrkB and total TrkB were further estimated by densitometric analyses (Figure 1B, C). Our results showed that TrkB phosphorylation was significantly enhanced in HH compared with the control ( $P < 0.05$ ) (Figure 1B).

The elevated phosphorylation of TrkB could be due to excessive expression of BDNF (the specific ligand for TrkB) in HH tissue. To test this, we compared the mRNA levels of mature BDNF between HH and control groups by real-time RT-PCR. As in Figure 1D, when normalized to the mRNA level of a housekeeping gene (GAPDH), the expression level of BDNF in HH was significantly higher than that in the control group ( $P < 0.01$ ).

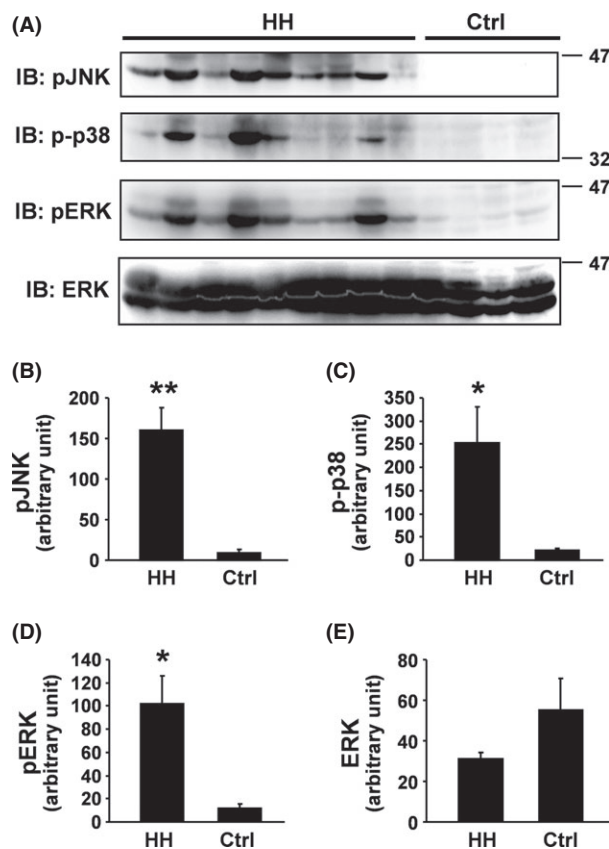
### Hyperactivation of MAPKs in HH Tissue

We next sought to assess the TrkB downstream signaling cascades. MAPKs are a family of serine/threonine kinases that play important roles in signal transduction initiated by RTKs including Trks [29]. MAPKs mainly include ERK1/2 (p44/p42 MAPKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK [29]. Thus, we examined the activation status of these three major MAPKs. Immunoblotting with anti-active p38 (p-p38), anti-active JNK1 (pJNK1), and anti-active ERK1/2 (pERK1/2), respectively, revealed that each antibody detected corresponding active forms of MAPKs present in HH tissue (Figure 2A). In contrast, the signals were almost negligible in all controls. Immunoblotting with anti-ERK antibody confirmed the equal protein loading (Figure 2A). Densitometric analyses further revealed that the respective pJNK1, p-p38, and pERK (but not ERK) levels were significantly higher in HH versus control tissue (p-p38,  $P < 0.05$ ; pJNK1,  $P < 0.01$ ; pERK1/2,  $P < 0.05$ ) (Figure 2B–E). These findings suggest that all three major MAPK pathways were highly activated in HH tissue.

### Interrelationship Between Active Akt and PTEN in HH Tissue

In parallel with the MAPK pathways, Akt is another key signaling molecule downstream of RTKs [30]. Akt is a serine/threonine kinase and its activity can be impacted by the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), which is upstream of Akt. PTEN is considered as the primary negative regulator of Akt [31]. Loss of PTEN or PTEN mutation is the most common cause of hyperactivation of Akt signaling in many human diseases [32]. Here, we assessed the Akt activation, PTEN expression, and their interrelationship by immunoblotting with anti-phospho-Akt (pAkt), anti-Akt, and anti-PTEN, respectively, combined with densitometric analyses (Figure 3). Our results showed that the pAkt (but not Akt) level was significantly higher in HH than that in the control group ( $P < 0.05$ ) (Figure 3A–C). Surprisingly, the PTEN level in HH was also higher than that in the control ( $P < 0.05$ ) (Figure 3A,D).

It is known that PTEN possesses a C-terminal, noncatalytic regulatory domain with three phosphorylation sites (Ser380, Thr382, and Thr383) that regulate PTEN stability and may affect its biological activity [33]. To confirm our findings of PTEN expression herein, we performed immunoblotting with an anti-phospho-PTEN (Ser380/Thr382/Thr383) (pPTEN) antibody (Figure 3A)

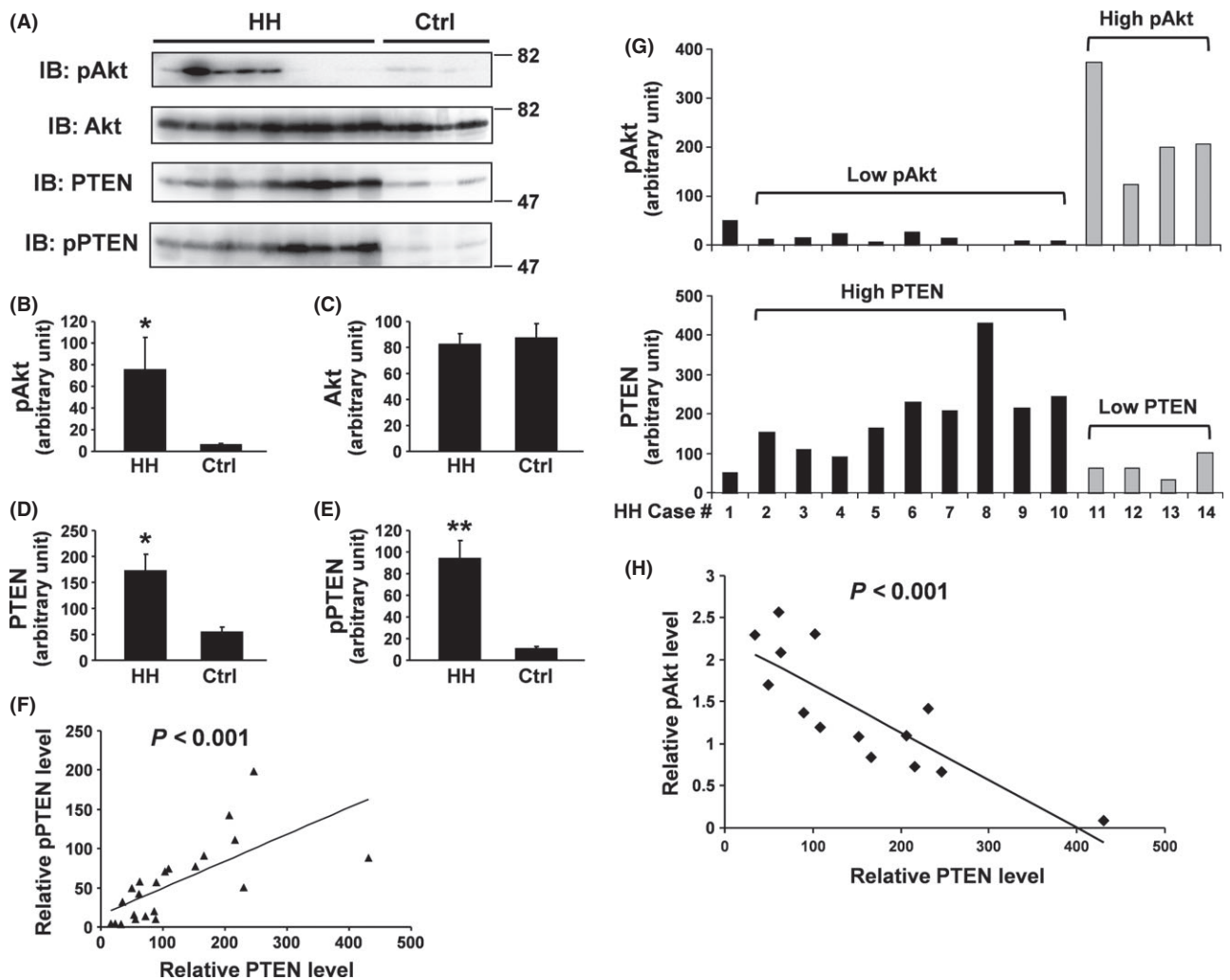


**Figure 2** Hyperactivation of MAPKs in HH. Proteins extracted from HH and control tissue specimens were analyzed by immunoblotting with anti-active JNK (pJNK), anti-active p38 MAPK (p-p38), anti-active ERK (pERK), and anti-total ERK, respectively (A). Densitometric analysis results of immunoblotting data of anti-pJNK (B), anti-p-p38 (C), anti-pERK (D), and anti-ERK (E), respectively, from 14 HH and eight controls are shown. \* $P < 0.05$ ; \*\* $P < 0.01$ .

and subsequent quantification (Figure 3E). The results indicated that the pPTEN level was indeed higher in HH tissue compared with the control (Figure 3E). Further, pPTEN positively correlated with PTEN ( $P < 0.001$ ) (Figure 3F). Collectively, our data suggested that for the comparison between the two groups (HH versus control), the pAkt level did not exhibit a negative relationship with the PTEN level as expected. Rather, both were higher in the HH group than those in the control group. However, when comparing the pAkt and PTEN levels within the HH group ( $n = 14$ ), we uncovered that pAkt displayed a negative relationship to PTEN. It seemed that the HH group could be classified into two subgroups, Low pAkt-High PTEN and High Akt-Low PTEN (Figure 3G). Correlation analysis further confirmed such an inverse interrelationship ( $P < 0.001$ ) (Figure 3H).

### Increased PLC $\gamma$ 1 Phosphorylation and CaMKII Expression in HH Tissue

The PLC $\gamma$ /CaMKII ( $\text{Ca}^{2+}$ /calmodulin-dependent kinase II) pathway is one of the major pathways that are activated upon BDNF binding to TrkB [16,17]. Functional expression of CaMKII

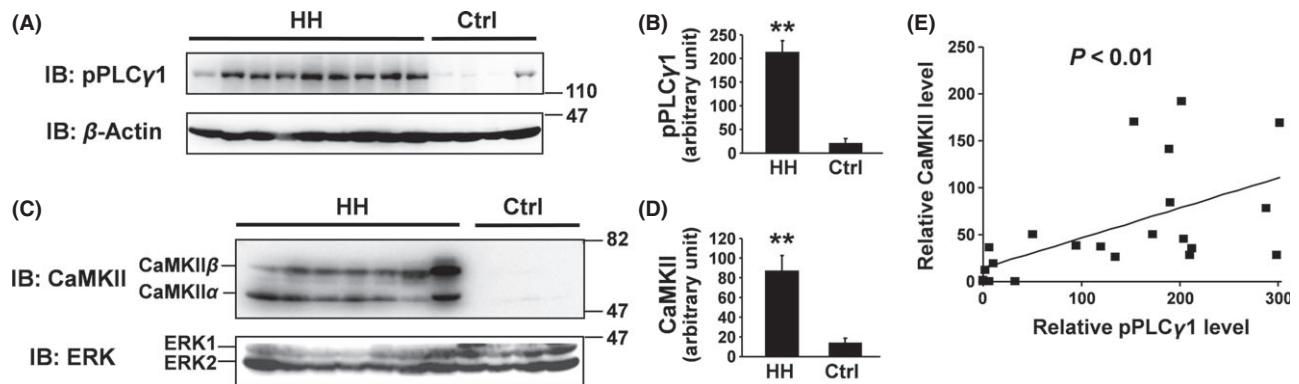


**Figure 3** Profiles of Akt activation and PTEN expression in HH. **(A)** Proteins extracted from HH and control tissue specimens were analyzed by immunoblotting with anti-phospho-Akt (pAkt), anti-total Akt, anti-total PTEN, and anti-phospho-PTEN (pPTEN), respectively. **(B–E)** Densitometric analysis results of immunoblotting data of anti-pAkt **(B)**, anti-Akt **(C)**, anti-PTEN **(D)**, and anti-pPTEN **(E)**, respectively, from 14 HH and eight controls are shown.  $*P < 0.05$ ;  $**P < 0.01$ . **(F)** Correlation analysis indicates a positive relationship between pPTEN and total PTEN levels. **(G)** Within HH group, pAkt levels show a negative relationship with PTEN levels. In this display, the HH subject No. 1 was used as a standard for the purpose of densitometric analyses with its mass set as 50. The HH subjects No. 2–10 had relatively lower pAkt but higher PTEN. Conversely, the HH subjects No. 11–14 had relatively higher pAkt but lower PTEN. **(H)** Correlation analysis confirms the negative relationship between pAkt and PTEN within HH group.

is regulated by  $Ca^{2+}$ /calmodulin complex downstream of PLC $\gamma$  [16]. The increased TrkB phosphorylation at Tyr816 (the docking site for PLC $\gamma$ ) in HH versus control tissue (Figure 1) prompted us to further assess this pathway by immunoblotting followed by densitometric analyses. The results indicated that PLC $\gamma$ 1 phosphorylation was significantly enhanced in HH versus control tissue ( $P < 0.001$ ) (Figure 4A,B). Our results also revealed that the anti-CaMKII (pan) antibody detected two isoforms of CaMKII, including CaMKII- $\alpha$  (~50Kd) and CaMKII- $\beta$  (~60Kd), and both were significantly upregulated in HH versus control tissue ( $P < 0.01$ ) (Figure 4C,D). As expected, correlation analysis confirmed the positive correlation between PLC $\gamma$ 1 phosphorylation and CaMKII expression ( $P < 0.01$ ) (Figure 4E).

### Downregulation of KCC2 in HH Tissue

It has been previously reported that the expression of the  $K^+$ - $Cl^-$  cotransporter KCC2 in adult rat neurons is downregulated following epileptiform activity and/or neuronal damage by activation of BDNF-TrkB signaling [21,22]. Here, we asked whether KCC2 expression was altered in HH in comparison with control tissue. The immunoblotting and densitometric analysis results indicated that the KCC2 level in HH tissue was significantly lower than that in the control ( $P < 0.01$ ) (Figure 5A,B). Likewise, immunostaining in tissue section slices demonstrated strong anti-KCC2 immunoreactivity in controls versus weak signals in HH tissue (Figure 5C, arrows).



**Figure 4** Enhanced PLC $\gamma$ 1-CaMKII signaling in HH. **(A and B)** Proteins extracted from HH and control tissue specimens were analyzed by immunoblotting with anti-phospho-PLC $\gamma$ 1 (pPLC $\gamma$ 1) and anti- $\beta$ -actin, respectively **(A)**. Densitometric analysis results of immunoblotting data of anti-pPLC $\gamma$ 1 from 14 HH and eight controls are shown **(B)**. **(C and D)** Proteins extracted from HH and control tissue specimens were analyzed by immunoblotting with anti-CaMKII and anti-ERK, respectively **(C)**. Densitometric analysis results of immunoblotting data of anti-CaMKII from 14 HH and eight controls are shown **(D)**. **\*\* $P$  < 0.01**. **(E)** Correlation analysis indicates a positive relationship between CaMKII and pPLC $\gamma$ 1 levels.

### Pharmacological Manipulation of TrkB Signaling Alters HH Neuronal Firing Rate

As above, we have demonstrated that the BDNF-TrkB signaling is upregulated, and concomitantly, KCC2 expression is downregulated in HH compared with the control tissue. These findings suggest that activation of BDNF-TrkB signaling may underlie the KCC2 downregulation and thereby GABA-mediated excitation in HH. If this is true, HH neuronal firing would be altered if the BDNF-TrkB signaling is activated or blocked.

We tested this possibility using patch-clamp recordings in freshly prepared HH slices. As shown in Figure 6A, patch-clamp current-clamp recording showed that a large-sized HH neuron exhibited spontaneous, irregular action potential (AP) firing at a low firing rate (<3 Hz). Bath application of BDNF (10 ng/mL) increased the HH neuronal firing (Figure 6B). In five large HH cells tested (from five HH patients), 2 min exposure to BDNF increased AP firing rate from  $2.02 \pm 0.75$  Hz to  $5.06 \pm 0.89$  Hz ( $P < 0.01$ , paired  $t$ -test). Then, we tested the effect of blockade of BDNF-TrkB signaling on HH AP firing. K252a is a selective inhibitor of Trk kinase activity [34], and has been widely used to verify BDNF/TrkB-mediated effects [15,35]. Our results showed that bath application of K252a (10  $\mu$ M) reduced the AP firing rate in a large (Figure 6C), but not in a small HH neuron (Figure 6D). Statistical analysis showed that exposure to K252a for 2 min reduced the AP firing from  $3.34 \pm 0.49$  Hz to  $1.98 \pm 0.33$  Hz ( $P < 0.01$ , paired  $t$ -test,  $n = 5$  from five patients). Together, these results suggest that the BDNF-TrkB signaling plays an important role in modulation of HH neuronal function.

### Discussion

The goal of our research is to develop a cellular and molecular model for seizure genesis within HH. Earlier work on surgically resected HH tissue focused on characterizing the phenotype, including electrophysiological properties, of the neurons within HH tissue [6–8,12,36]. We uncovered that most large HH neurons have the functionally immature property of depolarizing and

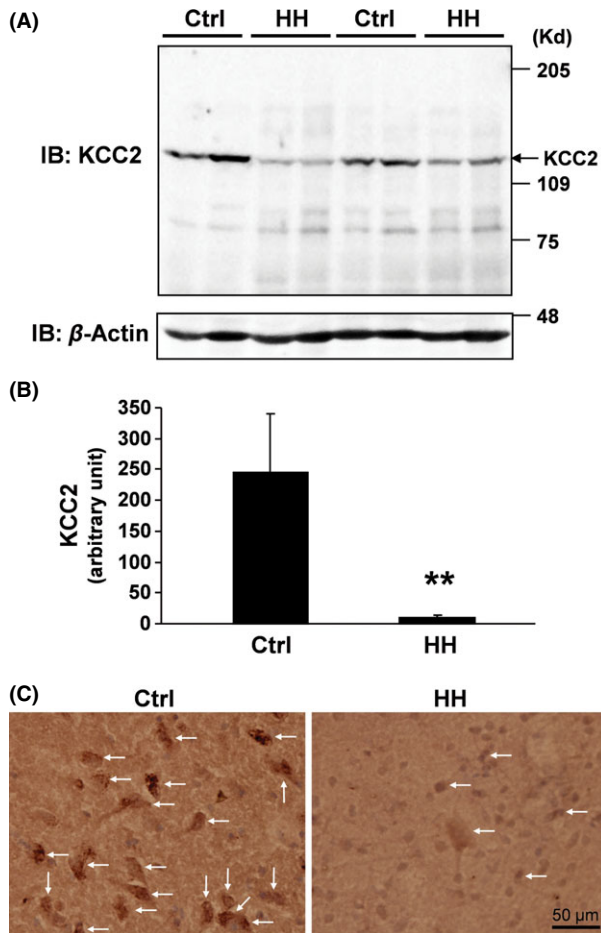
firing in response to GABA agonists as a result of elevated intracellular  $\text{Cl}^-$  concentration [6,12,13]. These neurons also show decreased KCC2 expression (relative to small HH neurons) at both mRNA and protein levels [6,12]. These data suggest that downregulated KCC2 expression may be causative for depolarizing and excitatory GABA responses in HH. In the present study, we explore intracellular signal transduction pathways potentially related to KCC2 downregulation in HH tissue. Our data show that compared with normal human hypothalamic control tissue, in HH tissue, (i) activation of TrkB and expression of mature BDNF are elevated; (ii) three major BDNF-TrkB signaling pathways, including MAPKs, Akt, and PLC $\gamma$ 1, are highly activated; and (iii) KCC2 expression is downregulated.

There is no animal model for HH, and consequently research on the cellular mechanisms resulting in epileptogenesis of HH relies upon the use of surgically resected human tissue. Obtaining control material is problematic, as there is a very high surgical premium on avoiding resection of adjacent tissue when resecting lesions within the hypothalamus. We make use of human autopsy control material as the best alternative under these circumstances. Our data suggest that protein expression and phosphorylation levels are stable during the postmortem time interval necessarily encountered for control tissue of this nature.

To our knowledge, this is the first report of comprehensive profiles of the BDNF-TrkB signaling system in HH, from the expression level of the ligand (BDNF) to phosphorylation/activation status of the receptor (TrkB) and its downstream signaling cascades. Our findings of increased BDNF mRNA levels and enhanced TrkB phosphorylation in HH tissue are consistent with other epilepsy models. It has been previously shown that BDNF is upregulated in multiple brain regions implicated in epileptogenesis, including hippocampus, neocortex, and ventromedial hypothalamus [37–39]. Similarly, seizures induced by hippocampal kindling lead to increased expression of TrkB, but not TrkA and TrkC, in the hippocampus [40]. Conditional neuron-specific deletion of TrkB prevents chronic seizures in the kindling model [41].

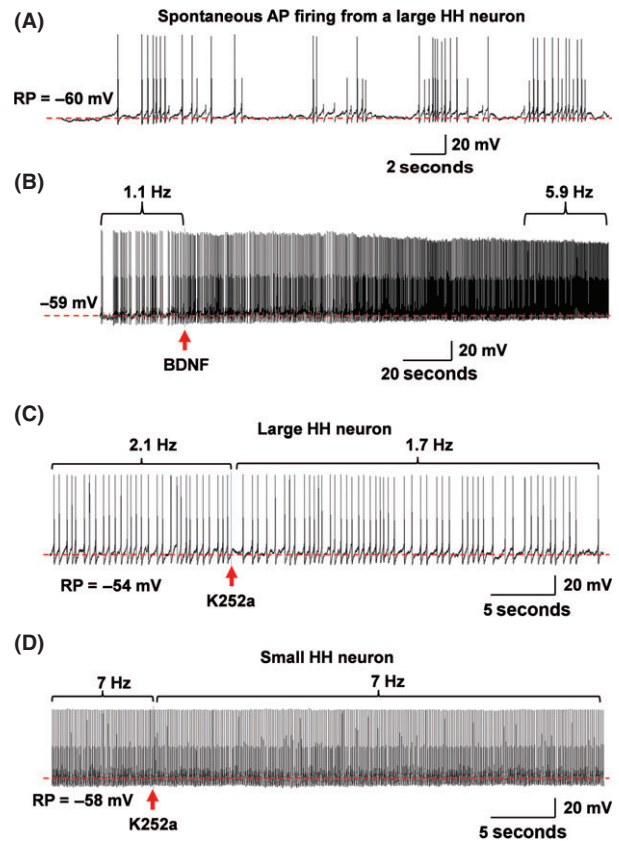
We have determined that multiple signaling pathways downstream to BDNF-TrkB binding are highly activated in HH tissue.





**Figure 5** Downregulation of KCC2 in HH. **(A and B)** Proteins extracted from HH and control tissue specimens were analyzed by immunoblotting with anti-KCC2 and anti- $\beta$ -actin, respectively **(A)**. Densitometric analysis results of immunoblotting data of anti-KCC2 from 14 HH and eight controls are shown **(B)**.  $**P < 0.01$ . **(C)** Immunostaining with an anti-KCC2 antibody in tissue sections (arrows) indicates downregulated KCC2 expression in HH versus control tissue. Bar, 50  $\mu$ m.

TrkB-mediated cellular changes are likely to occur via redundant activation of multiple pathways during normal development [42]. The details of the link between TrkB activation and downregulation of KCC2 expression are incompletely understood. It is now generally believed that BDNF binding to TrkB activates three major intracellular signaling pathways, including PLC $\gamma$ -CaMKII, Shc-Ras-MAPK, and Shc-PI3K-Akt cascades [16,17] (Figure S3). Rivera et al. reported that both PLC $\gamma$ - and Shc-mediated signaling cascades were required for BDNF-induced KCC2 downregulation [22]. Surprisingly, they showed that in *trkB<sup>PLC/PLC</sup>* mice with deletion of the PLC $\gamma$ -coupled pathway, BDNF rather caused upregulation of KCC2 [22]. The discrepancy of results from these studies could be related to the status of each or both of PLC $\gamma$ - and Shc-coupled signaling pathways in the context of the actual experimental settings. Rivera et al. suggested that downregulation of KCC2 seemed to take place if the Shc pathway was activated in conjunction with the PLC $\gamma$  pathway, whereas in the absence of



**Figure 6** Alteration of HH neuronal firing by pharmacological manipulation of BDNF-TrkB signaling. **(A)** Typical action potential (AP) firing from a large HH neuron showing spontaneous and irregular firing at low frequency as we previously reported [8]. **(B)** Bath application of BDNF (10 ng/mL) enhanced AP firing. **(A and B)** were recorded from the same neuron. **(C, D)** Blockade of TrkB signaling by K252a (10  $\mu$ M) reduced neuronal firing rate in a large **(C)**, but not in a small **(D)** HH neuron. **(A–D)** are representative traces from 10 HH neuron recordings (from 10 HH patients).

the PLC $\gamma$  signaling KCC2 upregulation was initiated by the Shc signaling [22]. In the current study, we demonstrate that compared with the control tissue, the Shc-mediated cascades (both Ras-MAPK and PI3K-Akt) and the PLC $\gamma$ -CAMKII cascade are highly activated in HH tissue. Concomitantly, KCC2 expression is downregulated. Although at this stage we cannot dissect these pathways in individual neurons or specific cell types within the tissue, our results mainly favor the opinion that both Shc- and PLC $\gamma$ -coupled pathways are involved in regulation of KCC2 expression.

We have hypothesized that BDNF-TrkB activation and downregulation of KCC2 expression contributes to epileptogenesis in HH tissue. However, other consequences of activating TrkB deserve consideration, since each of the activated intracellular signaling pathways has been associated with seizures and epilepsy. The ERK MAPK signaling pathway is a highly conserved kinase cascade that has been linked to epileptogenesis in several animal models [43–48]. In this study, we show that ERK1 and ERK2 are hyperactivated in HH tissue compared with the normal control.

Concomitantly, both BDNF expression and TrkB phosphorylation are enhanced in the HH tissue. Our results are in agreement with those from several previous studies. Nateri et al. reported that active ERKs are predominantly localized to the stratum lucidum in the hippocampus [48]. Immunohistochemical evidence indicated that although ubiquitously expressed throughout the brain, during epileptogenesis, TrkB undergoes phosphorylation at the mossy fiber synapse, and phosphorylated TrkB is specifically detected in the stratum lucidum [49,50]. The concomitant activation of TrkB and ERKs and their localization in the same anatomical region of the brain suggest that activation of the TrkB-Ras-ERK cascade is probably a key pro-epileptogenic signaling event.

The p38 and JNKs are also well-known MAPKs. Both p38- and JNK-directed signaling have been involved in pathological conditions including epilepsy, possibly via regulation of neuronal cell death [51–53]. However, compared with the BDNF-TrkB-Ras-ERK cascade, little is known about the roles of p38 and JNKs as downstream effectors of BDNF/TrkB in neuronal signal transduction [16]. We find increased activation of ERKs, p38 and JNK1 in HH versus control hypothalamus, suggesting that MAPK-regulated intracellular signals may participate in HH pathogenesis in some as-yet unknown manner.

The PI3K-Akt signaling pathway plays a vital role in determining cell fate and contributes to the onset and progression of many neurological disorders, including seizures and epilepsy [54,55]. Akt is a serine/threonine kinase that is constitutively inhibited by PTEN and has downstream effectors that include mammalian target of rapamycin (mTOR) [55–57]. Chronic hippocampal infusion of rapamycin (the mTOR kinase inhibitor) blocks mossy fiber sprouting in a rodent pilocarpine model of temporal lobe epilepsy [57]. In this study, we find that HH tissue levels of active Akt (pAkt) and total PTEN display a negative interrelationship (as expected). This suggests that a further classification of HH cases into two subgroups (Low pAkt-High PTEN and High pAkt-Low PTEN) is possible. The significance of this difference between different patients is unclear, but is noteworthy as other anatomical

or physiological features which differentiate HH tissue have been elusive.

Although the detailed mechanisms are still unclear, our data at least offer an explanation of how BDNF-TrkB signaling is linked to KCC2 downregulation and GABA<sub>A</sub> receptor-mediated neuronal excitation within the HH lesion. Based on our recent findings [6–8,36] and the new data presented in this study, we hypothesize that within the HH tissue, excessive BDNF activates TrkB, causing hyperactivation of multiple downstream signaling pathways, including the PLC $\gamma$ -CaMKII, the Shc-Ras-MAPK (ERKs, p38 and JNK), and the Shc-PI3K-Akt cascades (Fig. S3). These pathways may act independently or collaboratively to downregulate KCC2 expression, which is the key component for GABA-mediated excitation associated with gelastic seizures. Furthermore, we demonstrate that activation of BDNF-TrkB signaling (by BDNF) increase large HH neuron firing rate, while pharmacological inhibition of TrkB kinase activity markedly reduces the large, but not small, HH neuronal firing rate. Considering that large HH neurons have lower KCC2 expression and higher intracellular Cl<sup>-</sup> concentration [6], the BDNF-TrkB signaling appears to more specifically modulate large HH neuronal function, which supports our hypothesis.

## Acknowledgments

This work was supported by an Arizona Biomedical Research Commission Award (ADHS13-031259) and partially by a National Institutes of Health Award (R01GM085237). We thank the Harvard Brain Tissue Resource Center, the Human Brain & Spinal Fluid Resource Center (West Los Angeles Healthcare Center), and the University of Maryland for providing normal human hypothalamic tissue. We are grateful to our HH patients and their families for their willingness to allow scientific use of surgically resected tissue.

## Conflict of Interest

The authors declare no conflict of interests.

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## Supporting Information

The following supplementary material is available for this article:

**Figure S1.** Enhanced tyrosine phosphorylation in HH.

**Figure S2.** Postmortem interval (PMI) has no significant impact on protein phosphorylation and expression.

**Figure S3.** Proposed model of how BDNF-TrkB signaling contributes to KCC2 downregulation and GABA-mediated excitation and epileptogenesis.

**Table S1.** Lists of primary antibodies used in the study.