Decreased excitatory drive onto hilar neuronal nitric oxide synthase expressing interneurons in two chronic models of epilepsy

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Abstract

Excitation-inhibition imbalance of GABAergic interneurons is predisposed to develop chronic temporal lobe epilepsy (TLE). We have previously shown that virtually every neuronal nitric oxide synthase (nNOS)-positive cell is a GABAergic inhibitory interneuron in the denate gyrus. The present study was designed to quantify the number of nNOS-containing hilar interneurons using stereology in pilocapine- and kainic acid (KA)-exposed transgenic adult mice that expressed GFP under the nNOS promoter. In addition, we studied the properties of miniature excitatory postsynaptic current (mEPSC) and paired-pulse response ratio (PPR) of evoked EPSC in nNOS interneurons using whole cell recording techniques. Results showed that there were fewer nNOS-immunoreactive interneurons of chronically epileptic animals. Importantly, patch-clamp recordings revealed reduction in mEPSC frequency, indicating diminished global excitatory input. In contrast, PPR of evoked EPSC following the granule cell layer stimulation was increased in epileptic animals suggesting reduced neurotransmitter release from granule cell input. In summary, we propose that impaired excitatory drive onto hippocampal nNOS interneurons may be implicated in the development of refractory epilepsy.

Keywords: nNOS, Temporal lobe epilepsy, Interneuron, Synaptic transmission, Mouse models

Introduction

Temporal lobe epilepsy (TLE) is the most common type of adult pharmacoresistant partial epilepsy, characterized by abnormally excessive or synchronous neuronal activity in the hippocampus (Goldberg and Coulter, 2013). The imbalance of excitatory and inhibitory neurotransmission is accepted as the common mechanism of epileptic seizures. Moreover, the hippocampal dentate gyrus is considered by many to be the generator of TLE (Avoli, 2007). Pathological alterations include the loss of interneurons and synaptic reorganization of granule cells during epileptogenesis (Buckmaster et al., 2017).

An overwhelming body of clinical and experimental evidence suggests that hilar GABAergic interneurons are thought to act as a gate for runaway excitation (Goldberg and Coulter, 2013; Neumann et al., 2017). Silencing of hippocampal interneurons leads to the development of recurrent spontaneous seizures (Drexel et al., 2017). In particular, the vulnerability of subpopulations of GABAergic interneurons to seizure-induced damage has been extensively documented (Marx et al., 2013; Tóth et al., 2010; Tóth and Maglóczky, 2014). The hilar region encompasses a functionally heterogeneous population of GABAergic interneurons, such as the calcium-binding proteins (CaBP; parvalbumin, PV; calretinin, CR) and neuropeptides (somatostatin, SOM; neuropeptide Y, NPY)(Liang et al., 2013). Emerging evidence suggests there are fewer SOM- and NPY-containing hilar interneurons in animal models of epileptic seizures (Drexel et al., 2012; Huusko et al., 2015; Sun et al., 2007). A similar loss of PV- and CR-immunoreactive interneurons in response to status epilepticus (SE) was also documented in several studies (Drexel et al., 2017; Houser, 2014; Tóth et al., 2010). Collectively, these results confirm that hilar interneurons in the hippocampus may be more sensitive to seizure-induced damage.

Seizures are complex pathological network events characterized by abnormal synaptic transmission of GABAergic interneurons (Magloire et al., 2019; Righes

Marafiga et al., 2020). It has been reported that surviving hilar interneurons decrease excitatory synaptic input and become less active in epileptic animals (Sloviter et al., 2003). Previous data revealed that the reduction in the miniature excitatory postsynaptic current (mEPSC) rate of inhibitory neurons in rat models with recurrent seizures (Xiang et al., 2006). Studies within our laboratory have shown that neuronal nitric oxide synthase (nNOS)-positive cells are a major class of GABAergic interneurons in the dentate gyrus (Liang et al., 2013). Despite these observations, it remains unknown whether nNOS-positive cells are susceptible to seizure-induced injury, accompanied by alterations in excitatory transmission in nNOS-expressing interneurons.

Therefore, in the present study, we used stereological techniques to determine to nNOS-containing hilar interneurons counts in adult nNOS-Cre mice with chronic seizures induced by administration of pilocarpine and KA. Furthermore, we used hippocampal slices to obtain whole cell patch recordings from nNOS-positive cells in control and epileptic mice to compare their excitatory synaptic input. Our data show that there was a substantial loss of nNOS interneurons, indicating its vulnerability to seizure-induced damage. Moreover, down-regulation of mEPSC frequency and enhanced paired-pulse ratio (PPR) of evoked EPSC in chronically epileptic animals was also apparent. These results highlight additional findings towards the impaired excitatory drive onto nNOS interneurons in chronic seizure models and could be play a factor in the development of TLE.

Materials and methods

Animals

All experiments were conducted in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of the Zhengzhou University. Healthy adult male nNOS-Cre mice (22-25 g, Jackson Laboratories #017526 B6.129-$^{Nos1tm1(cre\_Mgmj/J)}$) were bred in house under a normal 12 h/12 h light/dark cycle and had ad libitum access to food and water.

Mouse models of chronic epilepsy

Pilocarpine treatment

The pilocarpine treatment was based on a modified version of a previous study (Chen et al., 2010). nNOS-Cre mice were treated with pilocarpine hydrochloride (280 mg/kg, i.p. Sigma-Aldrich, St. Louis, MO, USA) 30 min after atropine methylbromide (5 mg/kg, i.p.). Diazepam (10 mg/kg, i.p.) was administered 2 h after the onset of stage 4-5 seizures (Racine, 1972) and repeated if necessary to suppress convulsions. Approximately 70% of the treated mice experienced SE. Control mice consisted of animals that were treated with saline.

Intrahippocampal KA Model

The KA-induced chronic epilepsy model was established as previously described (Hedrick et al., 2017). In brief, mice were anesthetized with 1.5% isoflurane and placed in a stereotaxic frame (Stoelting Co, USA). A droplet of 50 nl of KA [20 mM KA diluted in phosphate-buffered saline (PBS) solution] was injected into right dorsal hippocampus (from the bregma: anteroposterior -2.3 mm, mediolateral -1.5 mm, dorsoventral -1.8 mm) using a 1 µl Hamilton syringe, mounted on an electric pusher (WPI, Sarasota, FL, USA). The injection needle was maintained within the cannula for 10 min and then slowly removed. All mice that received KA injections experienced SE, which was defined as three or more hours of continuous seizures classified as stage 4 or great seizures on a modified Racine Scale (Racine, 1972). Age-matched mice injected with PBS were used as controls.

Beginning one day after SE induction and for the next 60 days, the chronic epilepsy models were confirmed by the observation of at least one spontaneous recurrent seizure. Animals were then sacrificed for histological and electrophysiological changes in nNOS interneurons in experimental models of epilepsy.

Immunohistochemistry for nNOS-containing interneurons

Immunohistochemistry protocol for nNOS was conducted as we have previously

described (Wang et al., 2017). Briefly, mice were anesthetized with isoflurane and perfused transcardially with isotonic saline, followed by 4% paraformaldehyde in PBS (pH 7.4). After a 24 h period of post-fixation at 4°C, brains were cryoprotected in 30% sucrose in PBS. Right hippocampus was sectioned in the coronal plane at a nominal 40 µm thickness on a freezing microtome. Freefloating sections were washed in 0.1 M PBS to remove cryoprotectant, and endogenous peroxidases were quenched in 0.5% H_2O_2 in PBS. Sections were blocked with 5% normal horse serum in PBS with 0.5% Triton X-100. Sections were then incubated overnight at 4°C with goat anti-rabbit nNOS (1:4000, Sigma). Sections reacted with biotinylated secondary antibody goat anti-rabbit IgG (1:100, Vector Laboratories; Burlingame, CA, USA) for 2 h. Subsequently, the secondary antibody was detected with avidin-biotin-peroxidase complex (ABC Elite, Vector). The reaction product was visualized with 0.05% diaminobenzidine and 0.005% H_2O_2 substrate. After three rinses with PBS, sections were mounted on slides, dehydrated with increasing ethanol concentrations, rinsed in xylene and cover slipped with DPX mounting medium.

Stereological estimates of nNOS-immunoreactive interneuron counts

To determine the total number of nNOS-positive hilar interneurons in two mouse models of epilepsy, we utilized the optical fractionator probe as previously described (Wang et al., 2017). In brief, labeled cells were identified using a 60 × objective on a Nikon E600 series microscope (Nikon, Melville, NY, USA), and controlled using StereoInvestigator software (MBF Biosciences, Williston, VT, USA). Starting from a random point near the septal pole of the hippocampus, 1-in-5 series of nNOS immunostained sections were sampled, corresponding to a mean of twelve slices per animal. Contours were drawn around the denate hilus, which was determined by its border with granule cell layer and straight lines drawn from the ends of granule cell layer to the proximal end of the CA3 pyramidal cell layer. Size of the counting frame was 40 × 40 µm. The dissector height was 10 mm. Number (N) of nNOS-containing cells was calculated using the optical fractionator where $N = \sum Q * t/h * 1/asf * 1/ssf$, as in West *et al* (West et al., 1991).

Whole-cell patch-clamp recording

All recordings from epileptic animals were made at 60 days after SE. nNOS-Cre mice were anesthetized with halothane. The brain was removed quickly and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing 95 mM NaCl, 7 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.8 mM KCl, 26 mM $NaHCO_3$, 15 mM glucose and 50 mM sucrose (pH 7.3-7.4) when aerated with 95% O_2 and 5% CO_2. Hippocampal coronal slices, 400 μm thick, were cut with a vibratome in ACSF. Slices were stored in ACSF in an interface chamber at 32°C for at least 40 min before being transferred to the recording chamber. The recording solution was identical to the incubation solution except for (in mM): 127 NaCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose.

Recordings were made from single GFP-expressing cells located in the hilus, identified under fluorescent and infrared differential interference contrast (IR-DIC) video microscopy with a Zeiss Axioskop 2 FS plus microscope. Patch pipettes (3-5 M) were pulled from borosilicate glass (WPI, Sarasota, FL, USA) and filled with (in mM) 20 KCl, 120 K-gluconate, 20 HEPES, 0.5 Na_4EGTA, 2 $MgCl_2$, and 2 Na_2ATP (pH 7.2) for voltage-clamp recordings. Recordings were low-pass filtered at 2 kHz and sampled at 5 kHz. Cells were held at -60 mV under a voltage clamp mode to record miniature excitatory postsynaptic current (mEPSC). mEPSC was measured in the presence of tetrodotoxin (TTX, 1 mM), D-2-amino-5-phosphonovaleric acid (D-AP5, 50 mM, Sigma), and bicuculline (10 mM, Sigma) from 10 min after establishing the whole-cell configuration. Only nNOS interneurons with stable series resistances ≤ 30 mOhm were used for analysis. The recordings were analyzed only when there was no significant change in the frequency or the amplitude of mEPSC during the 5 min recording.

To evoke synaptic current, a monopolar glass stimulating electrode filled with ACSF was used. Stimulus pulses were delivered using a WPI stimulus isolation unit (Model 850A, New Haven, CT). Stimulating electrode was placed in the granule cell layer at 50-100 μm away from the soma of recorded nNOS-positive interneuron. The stimulation intensity was set to 200% of threshold values. Paired stimuli were

delivered at interstimulus intervals of 10 ms, 20 ms, 30 ms, 50 ms, 100 ms, 150 ms and 200 ms, respectively. The amplitudes of the first (P1) and the second (P2) EPSC were measured and the paired pulse ratio (PPR) was calculated as PPR = P2/P1. Recordings were performed using the multiclamp amplifier (Axopatch 700 B) and the pCLAMP 10 acquisition software (Molecular Devices, USA). Data analysis was done using Mini Analysis Software (Synaptosoft, Decatur, GA, USA).

Statistical analysis

Data were analyzed using GraphPad Prism 7.01 (GraphPad Software, San Diego, CA, USA). Student's *t* test analysis was applied whenever two groups were compared. A p value less than 0.05 was considered significant. Results are presented as mean ± SEM.

Results

Loss of nNOS-containing hilar interneurons

Human surgical specimens and experimental animal models of TLE have demonstrated the loss of interneurons in the hilus of hippocampus (Houser and Esclapez, 1996; Kobayashi and Buckmaster, 2003; Sun et al., 2007). This loss was true for nNOS interneurons in mice that experienced SE for 60 days. In accordance with previous reports (Armstrong et al., 2011; Perrenoud et al., 2012), the nNOS-expressing cells corresponded to strongly labeled cells displaying large somata. Visual examination of the images indicated that there were fewer nNOS-positive interneurons in epileptic mice than in controls. The total number of nNOS interneurons of saline-treated mice, estimated by stereologically counting nNOS-expressing cells, was $(5.23 \pm 0.33) \times 10^3$ ($N = 8$). Nevertheless, in the pilocarpine-induced epilepsy model, the number of nNOS-immunoreactive cells was $(3.51 \pm 0.25) \times 10^3$, a 32.89% reduction compared to controls ($N = 8$, $p < 0.01$, **Figures 1A, C**).

Similarly, we showed that the total number of nNOS-containing interneurons in the PBS group was $(5.21 \pm 0.28) \times 10^3$ ($N = 8$). In the intrahippocampal KA mouse model, the number of nNOS-expressing cells was $(3.26 \pm 0.27) \times 10^3$, a

37.42% reduction as compared to the corresponding controls (N = 8, p < 0.001, **Figures 1B, D**). Together, our observations suggested that nNOS-positive hilar interneurons were highly vulnerable to seizure-induced injury.

mEPSC Rate but not amplitude is decreased in nNOS interneurons

To assess the global excitatory input to identified nNOS-expressing interneurons in normal adult and epileptic mice, mEPSC from GFP-labeled cells in the hilar region were recorded (**Figure 2A**). The mean rate of mEPSC recorded from GFP-positive interneurons in saline-treated mice was 4.21 ± 0.44 Hz (N = 8). The mEPSC frequency of pilocarpine-treated mice was lower when compared to saline-injected controls (2.62 ± 0.41 Hz, N = 9, p < 0.01, **Figure 2C**). In contrast, the average mEPSC amplitude was unaffected in saline-treated animals (16.23 ± 0.57 pA) as compared to pilocarpine -operated mice (15.32 ± 0.53 pA, p > 0.05, **Figure 2D**).

Similarly, whole cell recordings from nNOS-expressing interneurons in KA-exposed mice and control slices were conducted. As illustrated in **Figure 2B**, the mEPSC rate was robustly decreased in epileptic mice as compared to the controls. On average, the frequency was 2.83 ± 0.28 Hz (N = 8) in KA-treated mice and 4.46 ± 0.34 Hz (N = 7) in controls (p < 0.01, **Figure 2C**). Whereas, the median amplitude (KA 14.86 ± 0.43 pA; control 15.70 ± 0.55 pA), of mEPSC did not show any substantial differences (p > 0.05, **Figure 2D**) between two groups. Overall, the data strongly suggested impaired excitatory drive to nNOS interneurons in chronic epileptic animals.

Reduced transmitter release probability of granule cell input contacting nNOS interneurons

It was then determined whether the release probability at the excitatory synapses contacting nNOS-expressing interneurons was decreased in mice at 60 days following pilocarpine and KA injection. Previous studies reported that axonal branching in dentate granule cells were located at the hilar nNOS interneurons dendrites (Armstrong et al., 2011; Shlosberg et al., 2012). As an estimate for release

probability we evaluated the PPR of evoked EPSC from granule cell input. Pairs of EPSC were evoked at different interstimulus intervals within the granule cell layer adjacent to the nNOS interneuron that was voltage clamped at -60 mV. The PPR of granule cell input-evoked responses was significantly higher in GFP-labeled cells recorded from epileptic animals (N = 6) as compared to the controls (N = 7), when stimuli were delivered at intervals of 50 ms and 150 ms ($p < 0.05$, $p < 0.01$, **Figures 3 A-B**).

In intrahippocampal KA injection mode, paired stimulation of granule cell input delivered at 100 ms and 150 ms interpulse intervals produced the potentiation of the second EPSC in epileptic animals (N = 6 per group, $p < 0.01$, $p < 0.01$, **Figures 3C-D**). Consequently, the above results suggest that the increase of the PPR in epileptic mice reduced the release probability at excitatory synapses between granule cell input and hilar nNOS-expressing interneurons.

Discussion

The principal findings of this study indicate that the number of nNOS-positive hilar interneurons was reduced in two well-established mouse models of TLE. Moreover, we found reduced excitatory drive onto surviving nNOS-containing interneurons illustrated by the decreased frequency of mEPSC and increased PPR of evoked EPSC, in chronically epileptogenic slices in nNOS interneurons. These abnormal alterations of nNOS interneuorns may contribute to the development of recurrent seizures.

GABAergic interneurons in the dentate gyrus are predisposed to function as gatekeepers for epilepsy (Weissinger et al., 2017). Amounting studies suggest that the vulnerability of hilar interneurons is evident in numerous animal models and human patients with TLE (Cameron et al., 2019; Marx et al., 2013; Tóth et al., 2010; Tóth and Maglóczky, 2014). More specifically, epileptogenic pathological changes might disinhibit the hippocampal circuitry. The pathological changes include alterations in $GABA_A$ receptor subunit composition or properties (Doherty and Dingledine, 2001), death of GABAergic interneurons (Botterill et al., 2017; Zhang et al., 2009) and reduction in number of glutamic acid decarboxylase 67 (GAD67)-positive cells

(Botterill et al., 2017). It is well established that mice subjected to pilocarpine and KA administration can be used to model the complexity of epilepsy symptoms (Lévesque and Avoli, 2013; Nirwan et al., 2018). Therefore, it was first ascertained by stereology that the number of nNOS-containing interneurons was altered in two chronic models of TLE. Of particular note, we observed the substantial decline in nNOS-expressing cells from epileptic animals, which coincided with prior results observed in the decreased quantify of SOM and nNOS interneurons in individuals, pilocarpine- and KA-treated animals with TLE (Choi et al., 2007; Hofmann et al., 2016; Leite et al., 2002; Zhang et al., 2009). Additional research is needed to evaluate whether decreased nNOS interneuron number is dependent on time after SE.

To determine the functional impact of the loss of nNOS-containing interneurons in epileptic mice, the excitatory input to hilar nNOS interneurons which was examined by identifying the expression of GFP under the nNOS promoter. This method labels a representative sub-population of GABAergic interneurons regarding their neurochemical, electrophysiological and morphological features (Tricoire et al., 2011). Results showed that pilocarpine and KA treatment induced a significant decrease in mEPSC frequency but not amplitude in nNOS-positive cells in mice. These results demonstrated a decrement in global excitatory drive onto identified nNOS-expressing interneurons of chronic epilepsy. The data was in line with studies showing loss of excitatory synaptic input onto GABAergic interneurons in electrically stimulated seizure-induced animals (Bekenstein and Lothman, 1993; Sloviter, 1991). Thus, the decreased mEPSC rate in nNOS-expressing cells in epileptic animals could impair the excitatory drive to nNOS interneurons.

Further tests were performed to determine if the release probability of neurotransmitters from granular cell input terminating onto nNOS-positive hilar interneurons may be altered. PPR is known to be inversely correlated with the probability of neurotransmitter release (Oleskevich et al., 2000). Herein, it was discovered that the PPR calculated for the synapses of primary granular cell input with hilar nNOS-containing interneurons in epileptic animals was increased compared to the controls, indicating a reduced release probability. Similarly, an important study

by Doherty and his colleague demonstrated that paired-pulse inhibition of granule cell population spikes in hilar interneurons was progressively impaired following pilocarpine-induced seizure, which represents the synapse dependent impairment in excitatory input onto interneurons during epileptogenesis (Doherty and Dingledine, 2001).

In summary, the data presented here in this study shows a decrease in nNOS interneuron number in the denate hilus of chronically epileptic animals. Intriguingly, excitatory input onto nNOS interneurons is diminished in nNOS-containing interneurons in the two canonical mouse models of TLE. The determination that GABAergic interneurons were lost in epileptic foci led to the hypothesis that GABAergic interneuron dysfunction could cause epilepsy (Spampanato and Dudek, 2017). Therefore, the current results reveal that future progress requires careful consideration of the issue of causality when inferring a relationship between impaired excitatory drive onto nNOS interneuron in hippocampus and the development of spontaneous recurrent seizures.

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Figure legends

FIGURE 1 | (**A-B**) Loss of neuronal nitric oxide synthase (nNOS)-containing hilar interneurons in mice at 60 days after pilocarpine (PILO) and kainic acid (KA) treatment. Some nNOS-immunoreactive interneurons have been pointed with arrow in the hilar region. The number of nNOS-expressing hilar interneurons was reduced in epileptic tissue (**C-D**). Scale bar represents 50 µm. g = granule cell layer; m = molecular layer; h = hilus. Student's *t*-test. $n = 8$. $^{\#\#}p < 0.01$ vs. saline-treated mice. $^{\#\#\#}p < 0.001$ vs. PBS-exposed mice. Values indicate means ± SEM.

FIGURE 2 | Decreased frequency of miniature excitatory postsynaptic current (mEPSC) from hilar GFP-labeled GABAergic interneurons of epileptic animals. (**A**) In the pilocarpine-induced epilepsy model, continuous recordings of mEPSC from typical cells in a control mouse (upper) and in an epileptic mouse (bottom). (**B**) In the intrahippocampal KA model, representative recordings of mEPSC in control (upper) and KA-treated mice (bottom). Summary graph of mEPSC frequency (**C**) and amplitude (**D**) in two animal models of TLE. Lower mEPSC frequency was observed in chronically epileptic mice. In contrast, mEPSC amplitude in mice following pilocarpine and KA treatment was similar to that of controls. Number of mice: 8 saline, 9 pilocarpine; 7 PBS, 8 KA. $^{\#\#\#}p < 0.01$ vs. saline-treated controls, $^{\#\#}p < 0.01$ vs. PBS mice, unpaired *t*-test. Data are presented as means ± SEM.

FIGURE 3 | Paired-pulse response ratio (PPR) for evoked excitatory postsynaptic current is increased in nNOS-GFP interneurons from epileptic tissue. (**A**) In the pilocarpine-based animal model of TLE, averaged traces of paired-pulse recordings following granule cell layer stimulation for 10 ms, 20 ms, 30 ms, 50 ms, 100 ms, 150 ms and 200 ms interpulse intervals. (**B**) Significant difference in PPR between control (n = 6) and epileptic nNOS interneurons (n = 7) at 50 ms interpulse interval was detected. (**C**) In mice KA treatment model, averaged traces of paired-pulse recordings following granule cell layer stimulation. (**D**) Statistical difference in PPR between control and epileptic nNOS interneurons at 100 ms interpulse interval was observed. PPR calculated as the peak amplitude of response 2/ response 1. Number of mice: 6 PBS, 6 KA. Error bars indicate SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. saline-treated controls, $^{\#\#}p < 0.01$ vs. PBS group, unpaired t-test. Data are presented as means ± SEM.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal breeding and experimental procedures were performed in accordance with the guidelines of the Animal Experiment Committee of Zhengzhou University.

Consent for publication

Not applicable.

Competing interests

None declared.