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Anti-seizure effect and neuronal activity change in the genetic-epileptic model rat with acute and chronic vagus nerve stimulation

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ABSTRACT

Background: VNS showed time-dependent anti-seizure effect. However, the precise mechanism of VNS in acute and chronic anti-seizure effect has not been fully elucidated. Noda epileptic rat (NER) is genetic epilepsy model rat which exhibits spontaneous generalized tonic-clonic seizure (GTC) approximately once per 30 h and frequent dialeptic seizure (DS). We performed acute and chronic VNS on NER to focus on the acute and chronic anti-epileptic effect and neuronal activity change by VNS.

Methods: We performed acute VNS (2 h) on 22 NERs (VNS, $n = 11$, control, $n = 11$), then subsequently administered chronic (4 weeks) VNS on 10 of 22 NERs (VNS $n = 5$, control $n = 5$). We evaluated the acute and chronic anti-seizure effects of VNS on GTC and DS by behavioral and electroencephalographical observation (2 h every week). We carried out double immunofluorescence for biomarkers of short-term (c-Fos) and long-term (ΔFosB) neuronal activation to map regions in the brain that were activated by acute (VNS $n = 6$, control $n = 6$) or chronic VNS (VNS $n = 5$, control $n = 5$). Furthermore, we performed chronic VNS (4 w) on 12 NERs (VNS $n = 6$, control $n = 6$) with long-term observation (8 h a day, 5d per week) to obtain an adequate number of GTCs to elucidate the time dependent anti-epileptic effect on GTC.

Results: Acute VNS treatment reduced GTC seizure frequency and total duration of the DS. Chronic VNS resulted in a time-dependent reduction of DS frequency and duration. However, chronic VNS did not show time-dependent reduction of GTC frequency. There were significant c-Fos expressions in the central medial nucleus (CM), mediodorsal thalamic nucleus (MDM), locus coeruleus (LC), and nucleus of solitary tract (NTS) after acute VNS. And there were significant ΔFosB expressions in the lateral septal nucleus (LSV), medial septal nucleus (MSV), MDM, and pontine reticular nucleus caudal (PnC) after chronic VNS. Any decrease in frequency of GTCs by chronic VNS could not be confirmed even with long-term observation.

Conclusion: We confirmed acute VNS significantly reduced the frequency of GTC and duration of DS. Chronic VNS decreased the frequency and duration of DS in a time-dependent manner. The brainstem and midline thalamus were activated after acute and chronic VNS. The forebrain was activated only after chronic VNS.

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1. Introduction

Up to 30% of patients suffering from epilepsy will be poorly controlled by maximal therapy, some of these patients are candidates for surgical intervention (Sander, 2003). The first-line treatment for drug-resistant epilepsy is resective surgery. Vagus nerve stimulation (VNS) can be considered as second-line treatment when resective surgery is contraindicated or ineffective. VNS showed time-dependent anti-epileptic effect (Chrastina et al., 2018). However, the precise mechanism of VNS in acute and chronic anti-epileptic effect has not been fully elucidated.

In this study, we used Noda epileptic rat (NER/Kyo (NER)). NER occurred spontaneously in the Wistar rat strain (Noda et al., 1998). NER exhibit spontaneous generalized tonic-clonic seizures (GTCs) associated with paroxysmal EEG discharges, approximately once per 30 h, starting at 2–4 months of age (Noda et al., 1998). Moreover, we observed dialeptic seizure (DS) in NER for the first time in this study. NER has no organic brain lesions (Noda et al., 1998). Rather, inheritance analysis revealed that the incidence of GTCs in NER is controlled by multiple genes (Maihara et al., 2000). These findings suggest that NER may serve as an animal model for idiopathic epilepsy in humans. Previous studies of acute VNS in animal models of seizures and epilepsy used electrical or chemical-induced seizure models (Aalbers et al., 2011). Studies of chronic VNS in genetic epilepsy models are scarce with only one study to date (Genetic absence epilepsy rats from Strasbourg, GAERS) (Aalbers et al., 2011; Dedeurwaerdere et al., 2005). The advantage of using genetic animal models of epilepsy is that they may simulate the clinical situation more closely than other epilepsy models (Aalbers et al., 2011).

The afferent cell bodies of the vagus nerve, residing in the nodose and the jugular ganglia, project to the nucleus of the solitary tract (NTS). The NTS sends direct and indirect projections to many regions, including the brain stem, the forebrain, and limbic structures (Aalbers et al., 2011; Nemeroff et al., 2006). We evaluated neuronal activities in these structures using c-Fos, an indicator of short-term neuronal activation, and ΔFosB, which reflects long-term neuroadaptations(McClung et al., 2004). c-Fos immunohistochemistry has been widely used as a mapping tool to identify cells activated by various stimuli(Kovács, 1998; Ohno et al., 2009). FosB, of the Fos protein family (c-Fos, FosB, Fra-1, and Fra-2), has a splice variant termed ΔFosB(Kovács, 1998). c-Fos messenger RNA (mRNA) is induced within a few minutes following acute c-*fos* expression-inducing challenges, with peaks appearing between 30 and 60 min (Kovács, 1998). Maximal c-Fos level occurs between one and three hours, and then it gradually disappears from the cell nucleus by four to six hours after treatment (Kovács, 1998). ΔFosB, on the other hand, shows a more delayed activation and persists longer (Kovács, 1998).

We first explored neuronal activities using c-Fos immunoreactivity at brain sites activated by VNS in Wistar rats as a wild-type model. Second, we treated NERs with acute (2 h) and chronic (4 w) VNS as an epileptic model and evaluated the anti-epileptic effects of VNS on GTC and DS by simultaneous behavioral and electrographical observation (2 h a day, one day per week, for four weeks). This was followed with double immunofluorescence staining of c-Fos and ΔFosB at potential brain regions involved in the VNS mechanism of action. Lastly, we performed chronic VNS (4 weeks) to NERs with prolonged observation (8 h a day, 5 days per week) to observe a statistically adequate number of GTCs to elucidation the anti-epileptic effect of VNS on GTC in NERs. We studied the acute and chronic anti-seizure effect of VNS to different seizure types (GTC, DS) in epilepsy model rat and checked the acute and chronic change of neuronal activities in the cites, those have been considered to be involved with the mechanism of VNS based on previous reports (Cunningham et al., 2008; Furmaga et al., 2012; Naritoku et al., 1995) and where our results in the experiment using Wistar rat showed significant c-Fos immunoreactivity.

2. Materials and methods

All the experimental protocols in this study were approved by the Experimental Animal Research Committee at Hiroshima International University.

2.1. Animals

Sixteen male Wistar rats (Charles River, Yokohama, Japan) weighing 220–230 g (eight weeks old) for Experiment I, 24 NERs (NBRp-Rat #0010, National BioResource Project-Rat, Kyoto University, Kyoto, Japan) weighing 180–230 g (eight weeks old) of either sex for Experiment II, and 12 NERs weighing 180–230 g (eight weeks old) for Experiment III were housed in groups and maintained in a temperature-controlled environment (23 °C), with a relative humidity of 55%, under an alternating 12-h light–dark cycle (light-on: 8:00 a.m.; light-off: 8:00 p.m.) until electrode implantation for VNS. After the surgery, each rat was housed individually with access to food and water ad libitum. The housing conditions and the animal care methods were maintained according to the NIH *Guide for the Care and Use of Laboratory Animals*.

2.2. Experiment I

2.2.1. Surgical procedures and stimulation parameters

Wistar rats anesthetized with pentobarbital (50 mg/kg, intraperitoneal) were implanted with cuff electrodes (KU-212-045Aa, Unique Medical, Tokyo, Japan) around the left vagal nerve and left carotid artery to prevent Wallerian degeneration by the damage of vagal nerve when it was dissected from carotid artery under aseptic conditions, as described previously(Dedeurwaerdere et al., 2004). The cuff electrodes consisted of a bipolar platinum/iridium needle (tip diameter: 150 μm). The bipolar stimulating cuff electrode was configured with the cathode as the proximal lead and the anode as the distal lead. These procedures were performed in the VNS-treated (n = 8) and control (n = 8) groups.

VNS was performed using an external stimulating current source (KS-402, Unique Medical) connected to the cuff-electrode. At 3–7 d after implantation, the left vagus nerve was stimulated for two h (one burst of 20 Hz with 250-ms pulse-width of 2.0-mA output current for 30 s, every 5 min) in eight conscious rats, while the other eight conscious rats served as sham-stimulated controls.

2.2.2. Immunohistochemistry

Thirty minutes after the two-hour vagus nerve or sham stimulation, rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and perfused intracardially with 0.1 M phosphate-buffered saline (PBS). After perfusion with 300–500 ml of PBS containing 4% paraformaldehyde, brains were removed and preserved in PBS containing 30% sucrose for 3–4 d. Each brain was sectioned in a cryostat for immunochemical assay. In practice, two or three sets of serial 40 μm coronal sections were processed for immunohistochemistry(Cunningham et al., 2008).

Separate sets of serial sections were stained for c-Fos immunoreactivity (rabbit anti-c-Fos Ab-5; Calbiochem, San Diego, USA), as previously described(Cunningham et al., 2008). For c-Fos immunohistochemical staining, sections were incubated with the primary antibody (1:20,000) for 72 h at 4 °C. The sections were then incubated with biotinylated horse anti-rabbit IgG (1:200 in PBS, BA-1100; Vector Laboratories, Burlingame, USA) for two hours at room temperature. Sections were then reacted with an avidin–peroxidase conjugate (Standard Vectastain Elite ABC Kit, PK-6100; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride liquid substrate (Peroxidase substrate kit, SK-4100; Vector Laboratories). Slides were subsequently mounted on gelatin-coated slides, air-dried for 1–2 d, and cover-slipped using Multi-mount 220 medium (FM22001; Matsunami Glass, Osaka, Japan).

Images of each slice, prepared under the same conditions, were

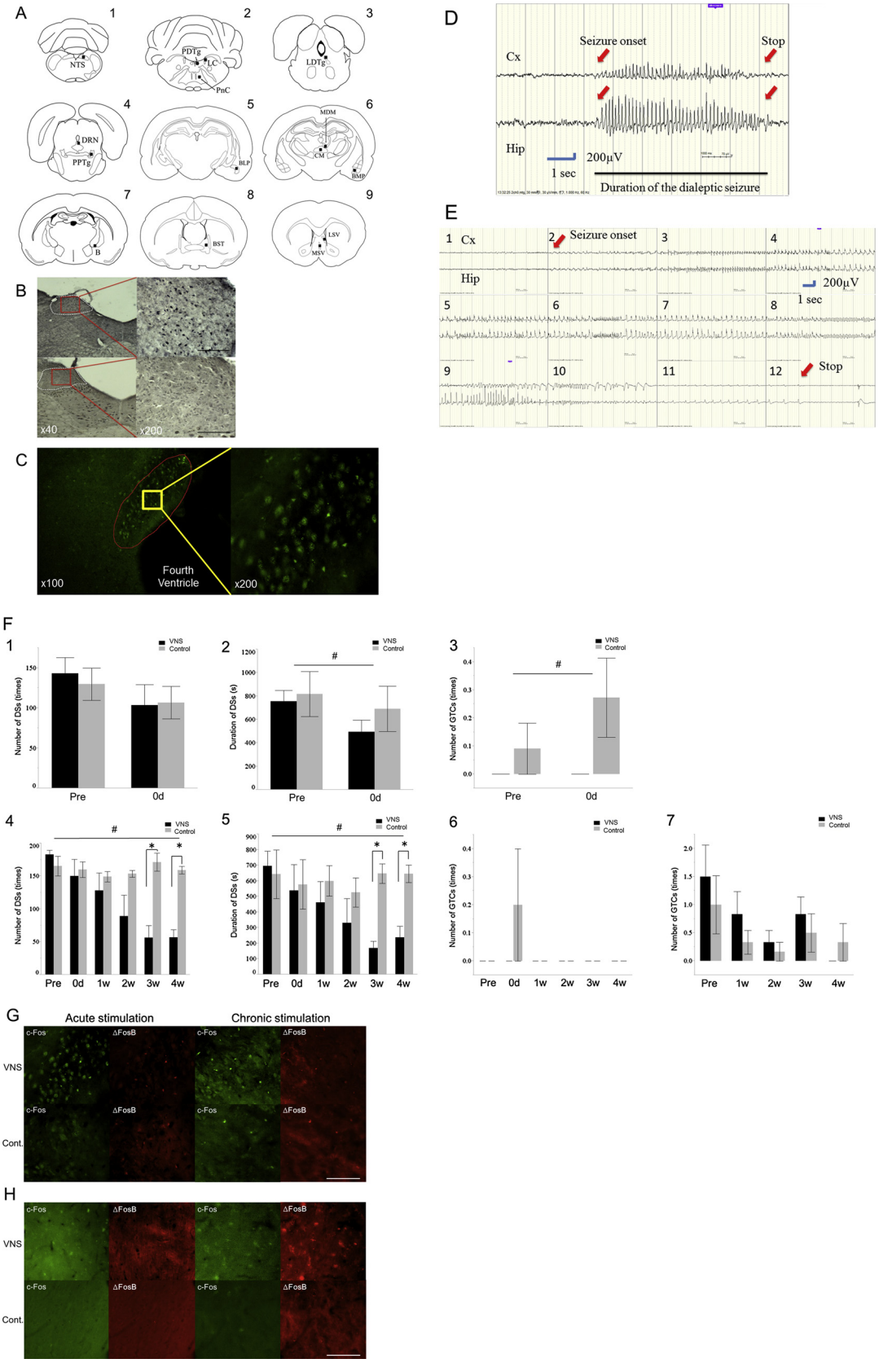

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Fig. 1. Fig. A. Schematic illustrations of the brain sections for quantitative analysis of c-Fos and ΔFosB-positive cells: Filled boxes in each section indicate the sample-areas analyzed. Each brain section of an anteroposterior coordinate (distance from Bregma) and targeted nuclei are as follows: A.1 (Bregma -13.30 mm) nucleus of solitary tract (NTS); A.2 (Bregma -10.04 mm) locus coeruleus (LC), posterodorsal tegmental nucleus (PDTg), and pontine reticular nucleus caudal (PnC); A.3 (Bregma -8.80 mm) laterodorsal tegmental nucleus (LDTg); A.4 (Bregma -7.80 mm) dorsal raphe nucleus (DRN) and pedunculopontine tegmental nucleus (PPTg); A.5 (Bregma -3.80 mm) basolateral amygdala nucleus (BLP); A.6 (Bregma -3.14 mm) basomedial amygdala nucleus posterior (BMP), central medial nucleus (CM), and mediodorsal thalamic nucleus (MDM); A.7 (Bregma -1.60 mm) nucleus basalis of Meynert (B); A.8 (Bregma -0.40 mm) bed nucleus of stria terminalis (BST); A.9 shows the lateral septal nucleus (LSV), and medial septal nucleus(MSV). Fig. B Representative images of c-Fos immunoreactive staining of NTS in Experiment I. Low- and high-power images (magnification 40× and 200×, respectively) of the region of interest (ROI) in the VNS-treated rats (upper) and control rats (lower). Scale bar: 100 μm. Fig. C. Example image for quantitative analysis of c-Fos positive cell in Experiment II. The low power field (left, 100x) shows LC (surrounded by red line). Square areas (red box in B, 300 μm × 300 μm; yellow box in C, 250 μm × 250 μm), at high magnification (right, 200×), on each ROI were quantified. Figure D and E. Representative ictal EEG findings during dialeptic seizure (D, DS) and generalized tonic-clonic seizure (E, GTC). Red arrows indicate ictal onset and end of DS or GTC. During DS, epileptiform discharges, consisting of 7–8 Hz repetitive spikes, were observed from cortex (Cx) and hippocampal (Hip) electrodes (D). Black bar in B illustrates the duration of the DS. During GTC, there are rhythmic fast activities over the cortex (Cx) and hippocampal (Hip) electrodes from seizure onset to end. The activities change into repetitive spike and wave bursts and build up during the seizure (E). Fig. F. Acute vagus nerve stimulation on 22 NERs (VNS $n = 11$, control $n = 11$) (1–3): Average number of dialeptic seizures (DSs) (F.1), generalized tonic-clonic seizures (GTCs) (F.3) and duration of DSs (F.2) before (Pre) and just after (0d) vagus nerve stimulation (VNS) during two h observation. Chronic vagus nerve stimulation on 10 NERs (VNS $n = 5$, control $n = 5$) (4–6): Average number of DSs (F.4), GTCs (F.6) and duration of DSs (F.5) at baseline (Pre), just after stimulation (0d), and the day after weekly VNS (or control) treatments (1 w, 2 w, 3 w, and 4 w), during the two hours observation. $*P < 0.05$, significant difference between VNS and control groups (paired t-test) at each observational time point. $\#P < 0.05$, significant time dependent interaction between groups (VNS vs control) in multivariate analysis of variance (MANOVA). Acute VNS suppressed the GTC (F.3) and reduced the duration of DS (F.2) significantly. Chronic VNS reduced the frequency (F.4) and duration (F.5) of DS.Chronic vagus nerve stimulation experiment on 12 NER (VNS $n = 11$, control $n = 11$) with long-term observation (F.7): Average number of GTCa for five days (8 h a day, total 40 h per a week) before stimulation (Pre), and for five days in 1st, 2nd, 3rd, and 4th week after starting VNS. Chronic VNS with long-term observation did not show a time-dependent anti-epileptic effect on the frequency of GTC in NERs (F.7). Black and gray columns represent the average number and duration of each seizures with and without VNS, respectively. Vertical lines on the columns indicate standard error. Figure G and H. Images of c-Fos and ΔFosB immunoreactive staining of the nucleus of LC (G), and MSV (H) in Experiment II. The upper images represent the c-Fos (green) and ΔFosB (red) staining of the region of interest (ROI) in the acute (left two columns of images) and chronic (right two columns) VNS-treated NERs respectively. The lower images represent the same fields as described above in the control rat. Scale bar: 100 μm.

acquired by light microscopy (BX-43, Olympus, Japan, digital camera; DS-Fi2, Nikon, Tokyo, Japan) at a magnification of 200× by an investigator who was blinded to the experimental treatment. From the images, square areas within a 300 μm × 300 μm grid focusing on each region of interest (ROI), were electronically excised with microscope imaging software (NIS Elements, ver. 4.1; Nikon, Tokyo, Japan), as shown in Fig. 1B. Immunoreactive cells in each ROI were manually counted with the aid of a computerized image analysis system (Image J, 1.42q, National Institutes of Health, Bethesda, USA). Briefly, immuno-positive cells were defined as those showing gray value < 50 (black–white: 0–255). The mean value determined from the number of immuno-positive cells in each ROI was obtained in each section per rat. ROIs were identified using HE-staining of serial section adjacent to each c-Fos stained section and the rat brain stereotaxic atlas of Paxinos and Watson(Paxinos and Watson, 1998). The nuclei of ROIs at each level of the brain were identified as in Fig. 1A.

2.3. Experiment II

2.3.1. Surgical procedures

The surgical procedure was described previously(Cunningham et al., 2008) and was similar to Experiment I. The coil electrode (Cyberonics Rat Lead Specifications, Cyberonics, TX, USA) was placed around the left cervical vagus nerve and left carotid artery and connected to a stimulator pack (NS-G103, Cyberonics, TX, USA). The stimulator pack was held in place by a sutured subcutaneous pouch created on the back of the rat. VNS were administered by an operational stimulator pack, programmed by a handheld computer (VNS-P201, Cyberonics). Controls received a dummy stimulator pack of the same size. For EEG recording, stainless-steel electrodes were stereotaxically implanted into the right dorsal hippocampus (3.0 mm posterior and 3.0 mm right lateral from bregma, 3.0 mm ventral from the cortical surface) according to the brain atlas of Paxinos and Watson(Paxinos and Watson, 1998). Three surface electrodes (stainless steel screws) were driven into the skull and placed: for recording from the right cortex (2.0 mm posterior and 2.0 mm right lateral from bregma), for grounding over the bone of the left frontal region, and over the left posterior cerebral cortex as a reference electrode. These procedures were performed in the VNS-treated and control groups.

2.3.2. Observation and recording of electroencephalogram (EEG)

Twenty-four NERs were randomly assigned to four treatment groups (each with $n = 6$) as follows: acute VNS, control acute treatment, chronic VNS after acute stimulation, and control treatment after acute stimulation. Two NERs were withdrawn from analysis due to post-surgical infection and device malfunction just after implantation: one assigned for chronic VNS and one assigned as a control for the chronic experiment. For acute VNS, two h of video observation (HDR-CX270 V, Sony, Tokyo, Japan) and EEG recording (BMSI5000, Nicolet, WI, USA) were performed 1–3 d before starting VNS (pre) and just after starting VNS (0day). For chronic VNS following acute stimulation, two h of observation and EEG recording were also performed at 1, 2, 3, and 4 weeks after starting VNS (total 8 h). On the day of the experiment, at 3–7 d after operating, we placed NERs individually in a plastic observation box (area: length 50 cm, width 40 cm, height 50 cm). Acute VNS consisted of one burst of 30 Hz with 500-ms pulse-width of 0.5-mA output current for 30 s every five min. We counted the number of DSs and GTCs, and the total duration of DSs for each recording. DS was defined as arrest with or without abnormal behavior corresponding with 7–8 Hz repetitive spikes (Fig. 1D). During DS, NER sometimes showed slow swinging their head left to right horizontally, and were unresponsive to visual stimuli, interrupted their ongoing activities, and often showed still posture just before the seizure initialization (Video A). GTC were accompanied by focal clonus, wild running and/or bouncing, brief opisthotonus, and were normally followed by a postictal flaccid stage after seizure cessation(Iida et al., 1998) as shown in Video B. Fig. 1E shows representative ictal EEG during GTCs. At the end of each experiment, 30 min after the final observation and EEG recording, their brains were removed and fixed as same in Experiment I for subsequent double immunofluorescence staining.

2.3.3. Double immunofluorescence staining of c-Fos and ΔFosB

Serial sets of 40 μm coronal sections from NER brains were prepared and processed for immunohistochemistry as done in experiment I. The primary antibody used does not discriminate between FosB and its splice variant, ΔFosB. However, we focused on long-term stimulation increasing ΔFosB levels, although a contribution from FosB cannot be excluded, as reported previously(Cunningham et al., 2008). To assess c-Fos and ΔFosB, sections were incubated with c-Fos (1:5000) (rabbit anti-c-Fos (sc-52)) and FosB (1:5000) (goat anti-FosB (sc-48-G); Santa

Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody for 72 h at 4 °C, after blocking with donkey serum (E27-18, Normal Donkey Serum Blocking Buffer, Golden Bridge International, Inc., WA, USA). The sections were then incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:1000) (A-21206) for c-Fos and Alexa Fluor 594 donkey anti-goat IgG (1:1000) (A11058, Thermo Fisher Scientific, Waltham, MA, USA) for FosB at room temperature for 4 h. The sections were washed in PBS, mounted on gelatin-coated slides, and cover-slipped using DAPI-containing mounting medium (ProLong Diamond Antifade Mountant with DAPI (P36966, Thermo Fisher Scientific). No immunoreactivity was detected with controls incubated with either primary or secondary antibody alone. The number of FosB-positive cells per section in selected brain regions was quantified by observers blind to the experimental conditions, as described previously(Cunningham et al., 2008).

For quantitative analysis, at least 2–3 representative sections from each brain region were imaged. Digital images were collected using an inverted microscope (IX81-ZDC, Olympus) equipped with a digital camera (DP30BW, Olympus). The software package MetaMorph (Molecular Devices, Sunnyvale, CA, USA) was used to control the system and for acquisition of the images. Square areas (250 μm × 250 μm, magnification 200; e.g. yellow box, Fig. 1C), were quantified from the ROIs indicated on the representative atlas schematic diagrams (based on brain stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 1998), Fig. 1A). Immunoreactive cells within ROIs of each slice were manually counted bilaterally with the aid of image analysis software (Image J, 1.42q).

2.4. Experiment III

Generally, NER approximately shows GTC once per 30 h(Noda et al., 1998). This experiment was designed to capture adequate number of GTCs for analysis.

2.4.1. Surgical procedures

As in experiment II, NERs were implanted with coil electrodes and either a VNS system (VNS group, $n = 6$) or a dummy stimulator pack (control group, $n = 6$). Our purpose in Experiment III was to observe GTC only (not DS), therefore no EEG electrodes were implanted to reduce noxious stress to NERs.

2.4.2. Observation and data acquisition

VNS parameters were the same as in Experiment II. During observations, NERs were placed individually in an observation box. Each observation consisted of eight hours of video recording (HDR-CX270 V, Sony), while an observer simultaneously counted GTCs behaviorally, without EEG recording. For five days in the first week after surgery, the total number of GTCs was counted and considered a baseline. The total number of GTCs was also counted on five days each week for four weeks after starting VNS. We compared the total number of GTCs in the baseline week with those in weeks one through four, after starting VNS.

2.5. Statistical analyses

Data from the cell counts in experiment I and II for each area of the brain in all groups, and the difference of seizure frequency (DS, DTC) and duration (DS) between VNS and control group at each observation point in experiment II and III were analyzed using paired t-test. The time dependent changes between groups (VNS vs control) in the number (DS, GTC) and the duration (DS) of each seizure type were assessed by multivariate analysis of variance (MANOVA) in experiments II and III. Data from the cell counts were analyzed by Steel-Dwass test.

All values are presented as the mean ± standard deviation. Differences were considered significant when $P < 0.05$. JMP Genomics 7.0 (SAS Institute Inc. NC. USA) was used for statistical calculations.

Table 1
c-Fos immunoreactivity following acute VNS in Wistar rat.

Structure	c-Fos positive cell		P value
	VNS	Control	
BMP	3.0 ± 2.4	1.6 ± 3.0	0.22
BST	0.99 ± 1.3	0.06 ± 0.12	*0.025
CM	6.5 ± 5.8	1.3 ± 2.1	*0.012
DRN	5.1 ± 5.8	2.1 ± 2.9	0.22
LC	7.4 ± 5.2	3.4 ± 4.8	0.064
LSV	4.3 ± 2.6	0.79 ± 1.7	*0.0044
NTS	21.1 ± 17.7	3.8 ± 3.6	*0.0054
PDTg	2.1 ± 2.3	1.1 ± 2.0	0.27
PPTg	3.1 ± 3.0	0.81 ± 1.3	0.21

All values of number of positive cells are presented as the mean ± standard deviation.
BMP, basomedial amygdala nucleus posterior; BST, bed nucleus of stria terminalis; CM, central medial nucleus; DRN, dorsal raphe nucleus; LC, locus ceruleus; LSV, lateral septal nucleus; NTS, nucleus of solitary tract; PDTg, posterodorsal tegmental nucleus; PPTg, pedunculopontine tegmental nucleus.
* Significantly different from controls, where $P < 0.05$ (Steel-Dwass test).

3. Results

3.1. Experiment I

Intermittent stimulation of the left VNS increased c-Fos immunoreactivity in four regions (Table 1). Specifically, c-Fos immunoreactivity was significantly increased in the thalamus (central medial nucleus; CM, $P = 0.012$), forebrain (lateral septal nucleus; LSV, $P = 0.0044$: bed nucleus of stria terminalis; BST, $P = 0.025$), and brainstem receiving afferent components of the vagal nerve (nucleus of solitary tract; NTS, $P = 0.0054$). Despite unilateral stimulation, equal-intensity, bilateral expression of c-Fos immunoreactivity was observed.

3.2. Experimental II

3.2.1. The acute and chronic anti-epileptic effects of VNS in NERs

We report the first observation of DS in NERs in this study. Epileptiform discharges consisting of 7–8 Hz repetitive spikes were observed during DS (Fig. 1D). In the acute VNS experiment, the number of DS (Fig. 1F.1), the number of GTCs (Fig. 1F.3) and the duration of DS (Fig. 1F.2) in the 11 NERs with 2 h VNS treatment were compared with those of 11 control NERs. Comparing time-dependent changes between VNS and control groups, acute VNS reduced the duration of DS [$F(120) = 4.561$, $P = 0.045$] and decreased the GTCs [$F(120) = 5.714$, $P = 0.027$] in frequency significantly. However acute VNS did not decreased DS frequency [$F(120) = 0.112$, $P = 0.742$] (Fig. 1F.1–3).

After acute stimulation, 10 NERs (VNS $n = 5$, control $n = 5$) were continuously stimulated for 4 weeks in a chronic experiment. During this chronic VNS, GTCs were observed only in the control group on day 0 (0.2 ± 0.2, 0d, Fig. 1F.6). NERs treated with chronic VNS showed a time-dependent anti-epileptic effect on the frequency [$F(1,8) = 1.196$, $P = 0.015$] and duration [$F(1,8) = 1.369$, $P = 0.011$] in DS compared to control groups (n = 5) (Fig. 1F.4,5); however, no anti-epileptic effect was seen on the frequency [$F(1,8) = 0.125$, $P = 0.347$] of GTCs in NERs treated with chronic VNS compared to controls (Fig. 1F.6). A significant difference between VNS and control groups in frequency (3week, $P = 0.001$; 4week, $P < 0.001$, Fig. 1F.4) and duration (3week, $P < 0.001$; 4week, $P = 0.002$, Fig. 1F.5) of DS was observed in and after 3 weeks of stimulation.

3.2.2. Immunoreactivity of c-Fos and ΔFosB after acute and chronic VNS in NERs

After 2 h of acute stimulation, VNS significantly increased c-Fos immunoreactivity in the CM ($P = 0.011$), locus coeruleus (LC,

Table 2
c-Fos and ΔFosB immunoreactivity following acute & chronic VNS in NER.

	Acute stimulation						Chronic stimulation					
	c-Fos positive cell			**ΔFosB** positive cell			**c-Fos** positive cell			**ΔFosB** positive cell		
Structure	VNS	Control	P value	VNS	Control	P value	VNS	Control	P value	VNS	Control	P value
B	2.7 ± 1.7	0.83 ± 0.94	0.12	1.0 ± 1.3	0.77 ± 1.7	0.21	6.5 ± 2.3	4.5 ± 2.3	0.39	10.4 ± 6.5	7.5 ± 1.8	0.8
BLP	2.8 ± 3.1	2.2 ± 2.3	0.82	0.17 ± 0.29	3.8 ± 5.2	0.18	13.1 ± 6.0	7.5 ± 2.1	0.18	21.9 ± 13.9	14.8 ± 4.7	0.54
BST	1.8 ± 1.8	0.5 ± 0.43	0.38	0.25 ± 0.25	0.29 ± 0.48	0.85	10.5 ± 6.6	4.8 ± 2.5	0.11	19.9 ± 17.9	9.5 ± 2.4	0.39
CM	4.8 ± 3.8	0.13 ± 1.3	*0.011	0.78 ± 0.89	0.27 ± 0.6	0.27	9.3 ± 6.1	5.5 ± 2.4	0.39	23.8 ± 17.8	7.6 ± 2.0	0.11
DRN	5.3 ± 4.0	3.2 ± 2.6	0.52	1.42 ± 1.4	0.17 ± 0.37	0.091	9.4 ± 5.3	5.0 ± 1.1	0.066	14.7 ± 11.0	5.9 ± 1.9	0.066
LC	7.4 ± 4.9	0.22 ± 0.25	*0.028	0.56 ± 0.51	0.00 ± 0.00	0.12	7.9 ± 3.7	4.4 ± 0.57	0.11	11.2 ± 7.9	6.3 ± 3.3	0.11
LDTg	4.1 ± 4.4	0.9 ± 1.2	0.12	0.72 ± 0.95	0.05 ± 0.11	0.24	4.8 ± 1.4	3.5 ± 1.0	0.22	8.5 ± 4.7	4.6 ± 1.3	0.11
LSV	3.8 ± 3.7	0.8 ± 1.1	0.14	1.47 ± 1.54	0.53 ± 0.51	0.34	7.1 ± 3.6	5.1 ± 2.6	0.39	15.2 ± 2.9	9.5 ± 2.5	*0.037
MDM	2.4 ± 2.7	0.23 ± 0.52	*0.049	0.94 ± 1.7	0.07 ± 0.15	0.16	7.1 ± 2.8	3.1 ± 1.8	0.085	19.3 ± 6.6	8.3 ± 1.9	*0.02
MSV	3.4 ± 2.3	1.5 ± 0.92	0.21	0.5 ± 0.69	0.47 ± 0.95	0.66	4.3 ± 2.7	4.5 ± 2.4	1	14.2 ± 6.2	6.4 ± 1.5	*0.02
NTS	5.2 ± 3.9	0.11 ± 0.19	*0.036	0.75 ± 1.0	0.06 ± 0.1	0.13	11.7 ± 8.4	6.1 ± 4.4	0.11	20.2 ± 10.8	10.7 ± 8.8	0.18
PDTg	6.3 ± 5.0	0.36 ± 0.38	0.053	2.1 ± 4.6	0.28 ± 0.25	1	5.1 ± 1.3	3.8 ± 0.6	0.14	7.2 ± 1.5	4.8 ± 1.3	0.27
PnC	3.4 ± 2.2	0.42 ± 0.72	0.052	2.1 ± 2.3	0.92 ± 1.6	0.51	3.7 ± 0.96	2.3 ± 0.56	0.067	5.0 ± 0.83	3.5 ± 0.67	*0.02

B, nucleus basalis of Meynert; BLP, basolateral amygdala nucleus; BST, bed nucleus of stria terminalis; CM, central medial nucleus; DRN, dorsal raphe nucleus; LC, locus ceruleus; LDTg, laterodorsal tegmental nucleus; LSV, lateral septal nucleus; MDM, mediodorsal thalamic nucleus; MSV, medial septal nucleus; n, number; NTS, nucleus of solitary tract; PDTg, posterodorsal tegmental nucleus; PnC, pontine reticular nucleus caudal.

* Significantly different from controls, where $P < 0.05$ (Steel-Dwass test). All values of number of positive cells are presented as the mean ± standard deviation.

$P = 0.028$, Fig. 1G), mediodorsal thalamic nucleus (MDM, $P = 0.049$), and NTS ($P = 0.036$). However, there was no significant difference between VNS and control groups in ΔFosB immunoreactivity after acute VNS (Table 2). After chronic stimulation for four weeks, ΔFosB immunoreactivities were significantly increased in VNS-treated NERs compared to controls in the LSV ($P = 0.037$), MDM ($P = 0.02$), medial septal nucleus (MSV, $P = 0.02$, Fig. 1H), and pontine reticular nucleus caudal (PnC, $P = 0.02$). However, there was no significant difference between VNS and control groups in c-Fos immunoreactivity after chronic VNS (Table 2).

3.3. Experiment III

There was no significant difference between the number of GTCs in chronic VNS-treated and control groups on five days of each week (pre-treatment, weeks 1–4). Chronic VNS for four weeks did not show time-dependent reduction of GTCs frequency in NERs [$F(1,5) = 0.0039$, $P = 0.953$] (Fig. 1F.7).

4. Discussion

4.1. Anti-epileptic effect of acute and chronic VNS to NER

In our study using NERs, acute VNS significantly showed reductions in the frequency of GTC and duration of DS. Chronic VNS decreased the frequency and duration of DS in a time-dependent manner. On the other hand, it has been reported that there was no acute or chronic anti-seizure effect of VNS on absence seizures in GAERS(Dedeurwaerdere et al., 2005) which is another validated genetic epilepsy model. The differences in the results between this study and our study may be derived from differences in genetic and biological background. Powell et al. described a mutation in the $Ca_v3.2$ T-type Ca^{2+} channel gene (Cacna1h) in GAERS(Powell et al., 2009). GAERS have a selective increase in the Ca^{2+} current, with a low threshold of activation in the reticular nucleus of the thalamus neurons. This is related to the pathological increase in synchronization, resulting in generation of bilateral and synchronous spike and wave discharges in GAERS (Tsakiridou et al., 1995). The mechanism of absence seizure in GAERS seems to involve the thalamocortical network. In contrast, Ohno et al. suggested that GTC in NER are of forebrain origin and are evoked primarily by activation of the limbic and/or cortical seizure circuits (Ohno et al., 2009). Harada et al. reported that reduced activity of Kir4.1 channels in the amygdala is involved in limbic hyperexcitability in NER(Harada et al., 2013). GTC in NER might correspond to the limbic system; however, the mechanism of DS in NER has not been investigated.

We observed significant reduction of GTC after acute VNS. This may be due to statistical variability in the data of GTC. NER exhibit a GTC approximately once per 30 h (Noda et al., 1998). Moreover, we performed only 2 h electro-behavioral observation at each observational point every week in Experiment II. We could capture only one GTC at the starting point of VNS (0d) in ten chronic groups. In experiment III, we performed longer observations, however there were no significant difference in the frequency of GTCs between chronically stimulated and control rats. These results suggested that VNS may not show anti-epileptic effect to GTC. On the other hand, we could capture a number of DSs. Acute VNS showed significant reduction of VNS in duration, not in frequency. Chronic VNS showed time-dependent seizure reduction in frequency and duration. VNS may reduce seizure duration first, and then reduce seizure frequency in DS. The mechanism of DS in NER is not uncovered. Further experiment will be needed to elucidate how VNS reduce seizure duration and frequency of DS in NER.

4.2. Regional expression of c-Fos in Wistar rat

Here, we showed VNS significantly increased c-Fos immunoreactive staining bilaterally in the NTS, CM, LSV, and BST in experiment I following acute/short term VNS stimulation. Several studies of non-epileptic rats report c-Fos expression in several forebrain and the brainstem structures(Cunningham et al., 2008; Naritoku et al., 1995), following acute VNS, and significantly increased c-Fos, and ΔFosB staining in the NTS, DRN, and LC (including many other cortical and limbic areas of brain), following chronic VNS(Cunningham et al., 2008; Furmaga et al., 2012). Multiple DBS targets have been studied, including the thalamus(Rahman et al., 2010). Experiment I and these previous reports indicate that structures interacting with the nuclei relaying to monoaminergic neurons in the thalamus and limbic system may play an important role in the mechanism of VNS. Therefore, we selected relevant regions of interest for Experiment II based on the results of Experiment I.

4.3. The change of neuronal activity after acute VNS in NER

After acute stimulation, we observed significant c-Fos expression in

the CM, MDM, LC and NTS in NERs. The CM controls generalized seizure threshold and expression. Microinjections of a γ-aminobutyric acid (GABA)-receptor agonist into the CM have markedly facilitated myoclonic and clonic seizures(Miller and Ferrendelli, 1990). A previous LC lesion can block the anticonvulsant effect of VNS in rats(Krahl et al., 1998) and LC activity is critical for limiting the spreading and duration of seizures since damage to LC neurons is able to convert sporadic seizures into status epilepticus(Giorgi et al., 2004). The MDM is a critical participant in the generation of seizures elicited focally from piriform(Cassidy and Gale, 1998). The MDM also regulates limbic seizure propagation, as enhancement of a GABA-mediated system within the MDM protects against focally evoked limbic motor seizures(Cassidy and Gale, 1998). The midline thalamus (CM, MDM), and brainstem (LC) were involved in the acute treatment phase of VNS.

4.4. The change of neuronal activityafter chronic VNS in NER

Following chronic stimulation, we observed ΔFosB expression in the MDM, LSV, MSV, and PnC. Tracking of functional magnetic resonance imaging (BOLD fMRI) signals during focal clinical limbic system seizures in human shows increased activity in the lateral septum (Blumenfeld, 2014). Pilocarpine-induced chronic epilepsy in rats leads to an 80–97% reduction of GABAergic neurons in the medial and lateral septum, while relatively sparing the cholinergic and glutamatergic neurons(Garrido Sanabria et al., 2006). The medial septum serves as a node to convey brainstem cholinergic and GABAergic projections to hippocampus(Scarlett et al., 2004).

Stimulation of the septum inhibits parenteral penicillin-induced spiking in cat hippocampus(Sabatino et al., 1985). Injection of carbachol into medial septum of the rat produces increased hippocampal theta and inhibits seizures from pentylenetetrazol (PTZ) and electrical kindling(Miller et al., 1994). Suppression of hippocampal epileptiform activity from PTZ was also achieved by 4–8 Hz medial septal stimulation.

Our data and these evidences support that midline thalamus (MDM), and forebrain (LSV, and MSV) activated by the brainstem (PnC) may play important roles in the chronic treatment phase of VNS.

4.5. Limitations and future directions

In our study, we show acute and chronic anti-epileptic effects of VNS on GTC and DS in NERs. However, chronic VNS did not suppress GTCs in NERs. Since NER only have a seizure approximately once every 30 h(Noda et al., 1998), it is challenging to use this model to evaluate an anti-epileptic effect to GTC. In previous studies, acoustic priming (Iida et al., 1998) and electrical stimulation for hippocampal kindling (Ishimaru et al., 2009) were adopted to facilitate spontaneous GTCs. These facilitators might be necessary to obtain an adequate number of GTCs for elucidation of precise anti-epileptic effects by VNS.

We observed significant changes of neuronal activities in the brain of epileptic model rat with acute and chronic VNS. These changes may be involved in the mechanism of acute and chronic VNS. However, there was no connection between our two lines of experiments (i.e. electro-behavioral observation and immunohistochemistry). The reason is that Fos family protein can be activated by multiple type of stimuli including pain, noxious stress, and seizures other than VNS (Kovács, 1998). Further experiment using biomarkers which are more specific to VNS will be needed to elucidate the mechanism of acute and chronic VNS.

5. Conclusion

We confirmed acute VNS significantly suppressed the frequency of GTC and reduced DS duration. Chronic VNS decreased the frequency and duration of DS, in a time-dependent manner. The brainstem and midline thalamus were activated in both acute and chronic treatment phase of VNS. The forebrain was activated only after chronic VNS.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eplepsyres.2019.106159.

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