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RESEARCH REPORT

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Hippocampal activation of 5-HT$_{1B}$ receptors and BDNF production by vagus nerve stimulation in rats under chronic restraint stress

Hwa Chul Shin[1*] | **Byung Gon Jo**[1*] | **Chan-Yong Lee**[2] | **Kang-Woo Lee**[1] | **Uk Namgung**[1]

[1]Department of Oriental Medicine, Daejeon University, Daejeon, Korea

[2]Department of Microbiology and Biotechnology, Daejeon University, Daejeon, Korea

Correspondence
Uk Namgung, Department of Oriental Medicine, Daejeon University, Daejeon, Korea.
Email: unamgung@dju.kr

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Abstract

A growing body of evidence shows that the electrical stimulation of the vagus nerve can improve mental illness including depression. Here, we investigated whether the vagus nerve stimulation (VNS) is involved in regulating the responsiveness of hippocampal neurons in rats under chronic restraint stress (CRS). c-Fos protein signals were detected 2 hr after VNS in 5-HT$_{1A}$ receptor-positive neurons in the dorsal raphe nucleus (DRN) as well as in the nucleus tractus solitarius (NTS). Chronic VNS was performed on a daily basis for 2 weeks using an implanted microelectrode in rats that had undergone CRS for 2 weeks. We found that the levels of both 5-HT$_{1B}$ receptors and phospho-Erk1/2 were decreased in parallel in the hippocampal neurons of CRS animals and then increased to the baseline levels by chronic VNS. Hippocampal induction of 5-HT$_{1B}$ receptors and phospho-Erk1/2 by VNS was diminished after the injection of 5,7-dihydroxytryptamine (5,7-DHT), a neurotoxin of serotonergic neurons, into the DRN. Hippocampal production of brain-derived neurotrophic factor (BDNF) was also upregulated by VNS, but the treatment of 5,7-DHT abrogated the effects of VNS on BDNF induction. VNS in CRS animals improved the behavioral scores in forced swimming test (FST) compared to sham-stimulated control. Our results suggest that VNS-mediated serotonergic input via 5-HT$_{1B}$ receptors into the hippocampal neurons may activate BDNF pathway and improve depressive-like behaviors in CRS animals.

KEYWORDS
animal model, BDNF, depression, dorsal raphe nucleus, Phospho-Erk1/2

Abbreviations: 5,7-DHT, 5,7-dihydroxytryptamine; BDNF, brain-derived neurotrophic factor; CRS, chronic restraint stress; DRN, dorsal raphe nucleus; NTS, nucleus tractus solitarius; VNS, vagus nerve stimulation.

*These authors contributed equally.

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1 | INTRODUCTION

In the vagus nerve, afferent nerve fibers account about 80% and send sensory inputs from major internal organs to the neurons in the nucleus tractus solitarius (NTS). While some of these signals are transmitted into the vagal motor neurons in the brainstem for feedback regulation of internal organs, others are connected to the nuclei in the upper brain areas. Accumulating evidence indicates that the vagus nerve acts as a bridge connecting between the brain and gut including enteric nervous system (Han et al., 2018), which is termed as brain-gut axis, and the vagus nerve activity may be involved in neuroimmune modulation (Breit, Kupferberg, Rogler, & Hasler, 2018). Ever since the vagus nerve stimulation (VNS) was investigated initially for the control of epileptic seizure in dogs (Zabara, 1985a,1985b), it has been applied for the treatments of mental illness such as treatment-resistant depression, memory improvement, and epileptic seizures (Carreno & Frazer, 2017; Clark, Naritoku, Smith, Browning, & Jensen, 1999; George et al., 2000; Penry & Dean, 1990).

While there has been growing concern on clinical application of VNS for the last two decades, its use is largely empirical and is weakly supported by scientific evidence. Application of acute VNS in normal rats induced c-Fos signals in NTS, parabrachial nucleus, paraventricular nucleus of hypothalamus ,and locus coeruleus (Cunningham, Mifflin, Gould, & Frazer, 2008), suggesting the activation of ascending central pathways by VNS. Chronic VNS in rats activated adrenergic neurons in the locus coeruleus and serotonergic neurons in the dorsal raphe nucleus (DRN), and serotonergic nerve was required for VNS-mediated antidepressant-like effects (Furmaga, Shah, & Frazer, 2011; Manta, Dong, Debonnel, & Blier, 2009). Other studies provide evidence that VNS affects levels of depression-related biological parameters in human patients. For instance, the levels of adrenocorticotropic hormone (ACTH) in the serum, which were significantly increased by corticotropin-releasing hormone (CRH) challenge in chronically depressed patients compared with healthy control group, were reduced by VNS (O'Keane, Dinan, Scott, & Corcoran, 2005). It was also reported in human subjects that serum levels of some proinflammatory cytokines were associated with severity of depressive symptoms, and VNS was effective in regulating pro- and anti-inflammatory cytokines (Corcoran, Connor, O'Keane, & Garland, 2005; Suarez, Krishnan, & Lewis, 2003). However, in animal studies, acute or chronic VNS induced the expression of IL-1β in the hypothalamus and hippocampus and also increased the expression of CRH and ACTH leading to increased serum corticosterone levels (De Herdt et al., 2009; Hosoi, Okuma, & Nomura, 2000). Taken together, VNS may influence hormonal regulation through the hypothalamic-pituitary-adrenal axis and production of inflammatory cytokines in relation to depression or depression-like symptoms in both human and experimental animals.

Chronic restraint stress (CRS) is widely used to examine stress-induced depression-like behavior (Chiba et al., 2012) and related neurobiological factors including stress-related hormones and neurotransmitters in a brain (Toth & Neumann, 2013). We reasoned that electrical stimulation of the vagus nerve may activate neurobiological responses in the brain in CRS animals and contribute to behavioral improvement, as has been implicated in human patients of depression. So far, the effects of VNS have not been explored by using animal models of depression while there are several reports showing that the application of VNS in normal animals induced antidepressant-like and anxiolytic activities (Furmaga et al., 2011; Grimonprez et al., 2015; Krahl, Senanayake, Pekary, & Sattin, 2014). Here, we investigated VNS in CRS animals and tried to delineate the relationship between VNS-induced neural pathway and behavioral consequences.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Sprague-Dawley rats (male, 200–250 g) were purchased from Samtako (Seoul, Korea), and maintained in animal room with regulated temperature (22°C), 60% humidity, and a 12-h light and 12-h dark cycle. All protocols involving live and postoperative animal care were approved by the Daejeon University Institutional Animal Use and Care Committee, and were in accordance with the Animal-Use Statement and Ethics Committee Approval Statement for Animal Experiments provided by Daejeon University (Protocol number: DJUARB2014-036; Daejeon, Korea). A total of 102 rats were used for the present investigation. The number of rats assigned to individual experimental groups are listed in Table 1.

2.2 | CRS and VNS

We conducted acute and chronic VNS surgeries in rats. For VNS surgery, animals were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg) and the left vagus nerve was exposed around ascending carotid artery. Acute VNS surgery was performed by placing the exposed nerve on a bipolar hook electrode of nichrome wire (Olofsson et al., 2015). The electrical current (10 mA, 5 Hz with 5 ms of pulse duration, 5 min; A-M Systems Inc., Sequim, USA) was applied once and animal was killed 2 hr later for immunofluorescence analysis. Chronic VNS was applied to animals that had undergone CRS. CRS was given to rats as described previously (Shin et al., 2017). Briefly, rats were placed in a well-ventilated cylindrical shape of Plexiglas container for 6 hr (9:00 a.m.–15:00 p.m.) each day for 14 days. Following

TABLE 1 The number of animals used in the present study

Animal groups	Experiments	Western blotting	Histology		Behavioral tests	
			IFS	H&E	FST	OFT
Acute VNS	CTL + acute VNS		2			
Chronic VNS	CTL	4	2		10	8
	CRS + VNS	4	2		10	8
	CRS + Sham	4	2		10	8
	CRS + VNS + DMT	4		2		
	CRS + VNS + saline	4		2		
BDNF injection	CRS + BDNF				8	
	CRS + Vehicle				8	

FST, forced swimming test; H&E, hematoxylin and eosin staining; IFS, immunofluorescence staining; OFT, open field test.

CRS, animals were randomly assigned into chronic VNS- and sham-treated groups (CRS + VNS and CRS + Sham, respectively), together with untreated control group (CTL). For chronic VNS, a custom-built, coiled platinum nerve cuff electrode (Inner diameter; 0.75 mm; Microprobes, Gaithersburg, MD, USA) was implanted by referring to an experimental procedure described by Zhang et al. (2013). The nerve cuff was wrapped around the exposed nerve, and the platinum wire, which was placed between the skin and muscle around the neck, was extended all the way back to the neck where the end of the wire was connected to the stimulation unit (A-M Systems Inc., Carlsborg, WA, USA). After suture, animals were recovered from anesthesia and returned to animal room. Electrical stimulation of the vagus nerve was initiated 3 days after the implantation of the simulation electrode with the same stimulation condition as acute stimulation and was given for 14 days. For sham control, the left vagus nerves of CRS animals were exposed and then closed by suturing the skin.

2.3 | Focal administration of drugs

To remove serotonergic neurons in the DRN, we anesthetized rats with ketamine (80 mg/kg) and xylazine (5 mg/kg) and placed in the stereotaxic apparatus. The dorsal skin on the area of hindbrain was exposed and the skull was perforated by using a drill. Glass microcapillary tube filled with 5,7-dihydroxytryptamine (5,7-DHT) solution (5 mM in 0.9% saline containing 1% ascorbic acid, Chemodex, Gallen, Switzerland) was stereotaxically guided into the DRN (from the bregma to posterior: −8.0 mm; L: 0 mm; DV: −5.8 mm) (Paxinos & Watson, 1998) and drug solution containing 5,7-DMT was injected with a flow rate of 1 μl/min for 5 min by using a micropump (Pump 11 Elite; Harvard Apparatus, Holliston, MA, USA). The amount of injection of 5,7-DMT (4.8 μg) was similar to doses which were injected (4–5 μg) into raphe nucleus and hypothalamus of rats in previous studies (File, Hyde, & MacLeod, 1979; Kiianmaa & Fuxe, 1977; Vergnes & Kempf, 1982).

For the injection of BDNF into the hippocampal CA1 area, rats were anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg) and placed onto the stereotaxic apparatus. BDNF (500 ng/μl in 0.1% BSA and 0.9% saline, Sigma-Aldrich, MO, USA) or an equivalent volume of saline vehicle was bilaterally injected into the hippocampal CA1 area (coordinate, AP: −4.2 mm, ML: 2.0 mm, DV: 1.8 mm) (Alonso et al., 2005; Paxinos & Watson, 1998) with a flow rate of 0.25 μl/min for 2 min by using micropump (Harvard Instrument).

2.4 | Western blot analysis

Hippocampal tissues were rapidly dissected from rats and sonicated in triton lysis buffer (20 mM Tris; pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate; pH 7.14, 2 mM sodium pyrophosphate, 2 mM ethylenediaminetetraacetic acid, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μM benzamidine, 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride). The supernatant was taken after centrifugation at 8,050 g for 10 min at 4°C. Protein (15 μg) was resolved by SDS polyacrylamide gel electrophoresis and subjected to immunoblotting. Antibodies used were anti-5-HT_{1B} receptor (rabbit-polyclonal, 1:2000; Abcam, Cambridge, UK), p-Erk1/2 (mouse-monoclonal; rabbit-polyclonal, 1:2000, Cell Signaling Technology, Danvers, MA, USA), anti-BDNF (rabbit-monoclonal, 1:2000; Abcam), and anti-actin (monoclonal, 1:10,000; MP Bio, Aurora, USA) primary antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit; 1:1000; Santa Cruz Biotech, or anti-mouse; 1:000, Amersham Biosciences, Buckinghamshire, UK). Protein bands were quantified by densitometric analysis of scanned images of X-ray film by using the i-Solution software (Image & Microscope Technology, Daejeon, Korea).

2.5 | Immunofluorescence staining

Animals were killed with an overdose of ketamine and xylazine and transcardially perfused with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS). The brain was dissected and immersed in 15% sucrose solution overnight. Sagittal or coronal brain sections (10 or 20 μm thickness) were prepared using a Cryostat (Leica, Wetzlar, Germany) and thaw-mounted on gelatin-coated slides. Pretreatment, blocking, and antibody reactions were performed as described previously (Chang, Kim, & Namgung,2012). Primary antibodies used were anti-c-Fos (monoclonal, 1:400; Abcam), anti-5-HT_{1A} receptor (rabbit-polyclonal, 1:400; Abcam), anti-5-HT_{1B} receptor (rabbit-polyclonal, 1:400; Abcam), anti-phospho-Erk1/2 (mouse-monoclonal; rabbit-polyclonal, 1:400, Cell Signaling Technology), and anti-NF-200 (mouse-monoclonal, 1:400, Sigma-Aldrich) antibodies, and secondary antibodies were fluorescein-goat anti-mouse (1:400; Molecular Probes, Eugene, USA) and rhodamine-goat anti-rabbit (1:400; Invitrogen, Carlsbad, USA) antibodies. Nuclear staining was performed using Hoechst dye 33258 (bisbenzimide; 2.5 μg/ml; Sigma) for 10 min before the final washing step. Fluorescence images were visualized and captured by digital camera attached to fluorescence microscope (Nikon, Japan), and transferred to a computer for analysis by using i-Solution software (Image and Microscope Technology, Daejeon, Korea).

2.6 | Forced swimming test and open field test

Forced swimming test was performed as previously described (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013; Shin et al., 2017). Each rat was placed in a transparent cylindrical chamber (height 50 cm, diameter 25 cm) filled with water at 27°C (depth 40 cm). Twenty four hours before the test, animals were adjusted in water for 15 min. Swimming test was conducted for 5 min following an initial 2 min of adjustment in water, and the immobility time and climbing time were analyzed by using a Smart version 2.5 video tracking system (Panlab, Barcelona, Spain). Immobility time was determined by measuring the period during which animals kept maintaining the head above the water without any additional actions. Duration of climbing behavior was determined by measuring the time during which animals displayed upward-directed movements of the forepaws along the side of the swim chamber. FST was also performed in CRS + BDNF and CRS + vehicle groups of rats. CRS was given to rats for 14 days (9:00 a.m.–15:00 p.m.) by placing in a well-ventilated cylindrical shape of Plexiglas container for 6 hr for 14 consecutive days. Then 24 hr later, BDNF or saline was injected into the hippocampus as described above, and 24 hr later, CRS + BDNF and CRS + Vehicle groups of rats were subjected to FST. Animals were adjusted for 1 hr in a test room and placed in a transparent cylindrical chamber (height 50 cm, diameter 25 cm) filled with water at 27°C (depth 40 cm). Scores of immobility and climbing duration were measured for 5 min following an initial 2 min of adjustment in water.

For open field test (OFT), rats in three experimental groups (CTL, CRS + Sham, CRS + VNS) were prepared as described above and were subjected to the test as described previously (Cheng et al., 2017; Shin et al., 2017). Briefly, animals were adjusted by placing in a test room for 1 hr and placed into a square chamber ($70 \times 70 \times 40$ cm^3). Behavioral scores were measured for 10 min following an initial 5 min of adjustment. Spontaneous locomotor activity was measured for 10 min by monitoring the total distance of movement by using video tracking software (SMART 3.0; Panlab S.L.U.), and the frequency of rearing, a behavior that the rat stands upright on its hind legs, was counted for 10 min.

2.7 | Statistical analysis

Data were presented as mean ± standard error of mean (SEM). The number data among animal groups were compared by Student's t test or one-way ANOVA followed by Tukey's multiple comparison test (GraphPad Prism 7.00), and statistically significant differences were set at $p < 0.05$.

3 | RESULTS

To determine whether VNS affected the activation in ascending neuronal pathways in a brain, we prepared sagittal brain sections 2 hr after VNS and analyzed c-Fos protein signals by immunofluorescence staining. c-Fos signals were clearly induced in neurons of the NTS and the DRN (Figure 1). 5-HT_{1A} receptors are known to be expressed in neurons of the raphe nucleus and involved in regulating serotonergic neuronal function as an autoreceptor (Artigas, 2013). Our data show that c-Fos signals in the DRN were largely colocalized with neurons expressing 5-HT_{1A} receptors, suggesting the activation of serotonergic neurons in the DRN by VNS.

Serotonergic neurons in the raphe nucleus project axons into several forebrain areas including the hippocampus. To determine whether VNS affected serotonergic neuronal activity in the hippocampus in association with depression-like behavior, we compared production levels of hippocampal 5-HT_{1B} receptors among animal groups of CTL, CRS + Sham, and CRS + VNS. Levels of 5-HT_{1B} receptors were significantly decreased in CRS + Sham animals compared with untreated CTL ($p = 0.049$), and then elevated to a similar level as that of CTL ($p = 0.022$, CRS + Sham vs. CRS + VNS) (Figure 2a). Examination of the production of 5-HT_{1B} receptors in the hippocampal subfields revealed that clear signals were detected in CA1 area of control group and CRS + VNS

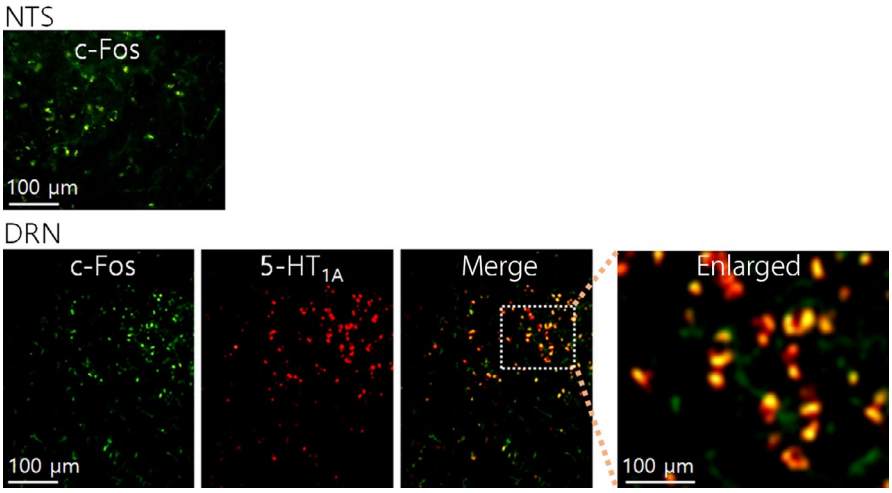

FIGURE 1 Immunofluorescence images of c-Fos and 5-HT_{1A} receptor signals in brain tissues. Sagittal brain sections were prepared from rats 2 hr after VNS and used for immunofluorescence staining by antibodies as indicated in the figures. DRN, dorsal raphe nucleus; NTS, nucleus tractus solitaries. [Colour figure can be viewed at wileyonlinelibrary.com]

groups, but the signal intensity was much reduced in CRS + Sham group (Figure 2b). Intense signals were seen in the soma of CA1 neurons and also in the stratum radiatum (arrows in Figure 2b). We also found that the levels of phospho-Erk1/2 protein in the hippocampus were significantly reduced in CRS + Sham group compared to control ($p = 0.0023$), and then upregulated by VNS ($p = 0.0013$, CRS + Sham vs. CRS + VNS) (Figure 2c). It was observed that, in both CTL and CRS + VNS groups, signals of phospho-Erk1/2 and 5-HT_{1B} receptors were highly colocalized in hippocampal CA1 neurons, but the signal intensities of both proteins were much weaker in CRS + Sham group (Figure 2d).

To further investigate whether serotonergic raphe neurons were required for the responses of hippocampal neurons in CRS + VNS animals, we injected a neurotoxin 5,7-DHT into the DRN area to destroy serotonergic neurons and investigated hippocampal proteins. Injection of 5,7-DHT into the DRN area of CRS + VNS animals resulted in a loss of many neurons in the DRN (Figure 3a). Levels of 5-HT_{1B} receptors and phospho-Erk1/2 in CRS+VNS animals were significantly decreased by 5,7-DHT injection compared with saline control ($p = 0.019$ and $p = 0.035$, respectively, Figure 3b,c). Previous studies reported that hippocampal production of BDNF is closely related to anti-depression-like effects in animal model of depression (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). BDNF level was significantly decreased in CRS animals compared to control animals and then elevated by VNS ($p = 0.035$ and $p = 0.012$, respectively, Figure 3d). Then, the treatment of 5,7-DHT suppressed BDNF induction by VNS ($p = 0.04$). Taken together, these data indicate that the activation of serotonergic neurons in the DRN may be involved in VNS-mediated activation of hippocampal neurons in terms of increases in 5-HT_{1B} receptor, phospho-Erk1/2, and BDNF.

To further investigate the effects of VNS on behavioral consequences, we conducted FST and measured the durations of immobility and climbing behaviors. Immobility time in CRS animals was significantly increased compared to control animals ($p = 0.0001$), and then decreased by VNS ($p = 0.0061$) (Figure 4a). Climbing duration was significantly decreased in CRS group compared with untreated control group and elevated by VNS (($p = 0.0007$ and $p = 0.005$, respectively) (Figure 4b). To determine whether or not the behavioral scores in FST were affected by animals' spontaneous locomotor activity, we analyzed the distance of movement and rearing behavior in OFT. Comparison of movement among CTL, CRS + Sham, and CRS + VNS groups did not show any significant differences ($p = 0.98$ for CTL vs. CRS + Sham; $p = 0.81$ for CRS + Sham vs. CRS + VNS; Figure 4c). Also, the measurement of rearing behavior showed no significant differences between CTL and CRS + Sham ($p = 0.36$) and CRS + Sham and CRS + VNS ($p = 0.75$) (Figure 4d), suggesting that the results obtained from the FST were not affected by their spontaneous locomotor activity.

To further examine whether hippocampal expression of BDNF mimics the antidepressant effect of VNS, we injected BDNF into the hippocampus of CRS animals and analyzed FST scores. There were some changes in immobility and climbing durations in BDNF-injected group compared to vehicle-treated group (14.3% decrease and 24.3% increase, respectively) but the differences were not statistically significant ($p = 0.11$ and $p = 0.23$, respectively, t test unpaired) (Figure 4e,f).

4 | DISCUSSION

The primary objective of current investigation was to identify molecular targets affected by VNS in the hippocampal neurons of CRS animals. As CRS is widely used to investigate depressive-like behaviors associated with persistent stress,

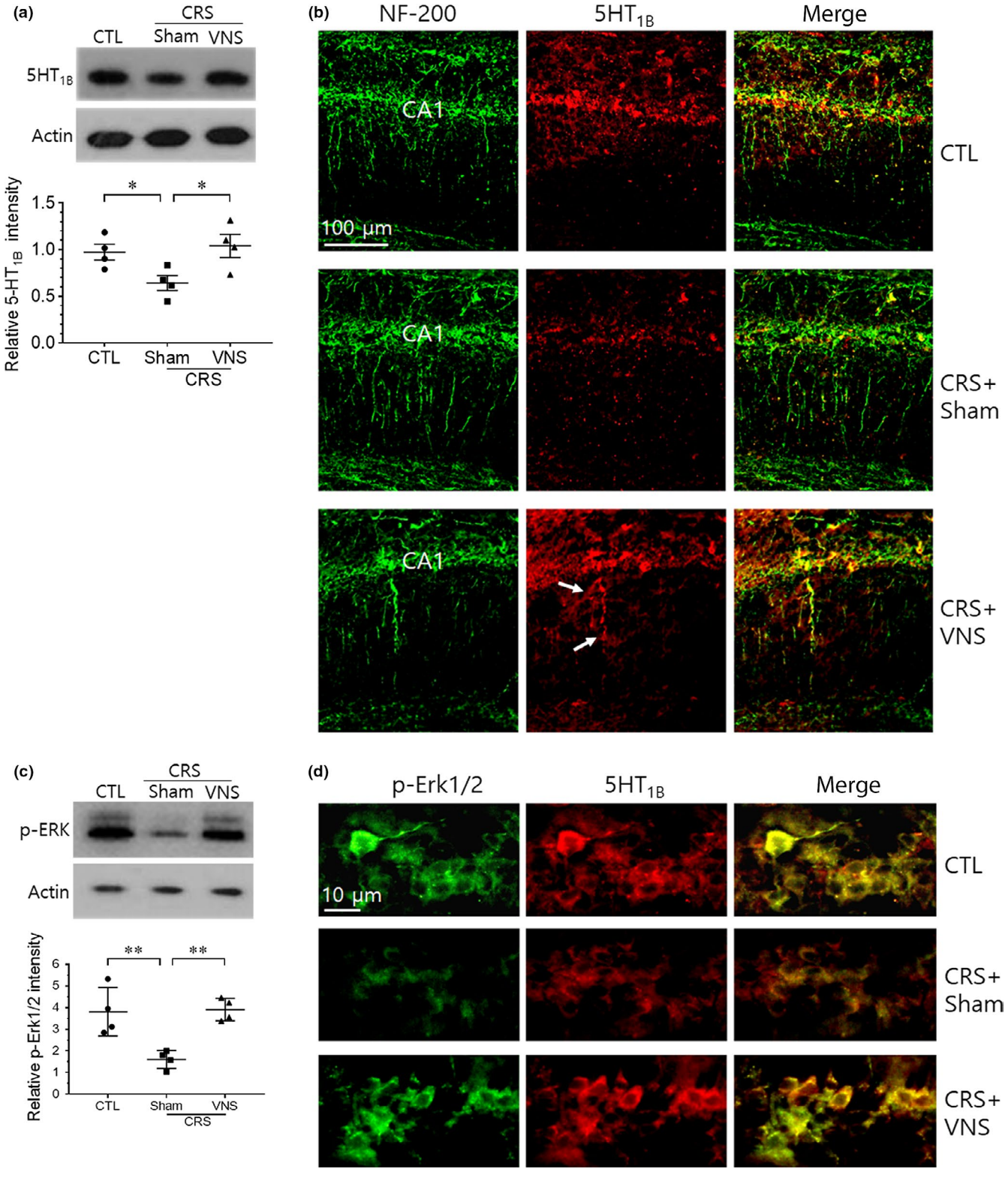

FIGURE 2 Regulation of 5-HT_{1B} receptors and phospho-Erk1/2 in hippocampal tissues by VNS in CRS animals. (a) Western blot analysis of 5-HT_{1B} receptors. Protein extracts were prepared from hippocampi in animal groups of control (CTL), CRS + Sham, and CRS + VNS. (b) Immunofluorescence images of 5-HT_{1B} receptor signals in hippocampal CA1 area of CTL, CRS + Sham, and CRS + VNS groups of animals. (c) Western blot analysis of phospho-Erk1/2. Protein extracts were prepared from hippocampi in animal groups of CTL, CRS + Sham, and CRS + VNS. (d) Immunofluorescence images of 5-HT_{1B} receptor and Erk1/2 signals in the hippocampal CA1 subfield of CTL, CRS + Sham, and CRS + VNS groups of animals. In (a) and (c), images in the upper panel are the representatives from four independent experiments, and quantification plots of relative protein band intensity to actin are shown in the lower panel. *$p < 0.05$, **$p < 0.01$ (One-way ANOVA). [Colour figure can be viewed at wileyonlinelibrary.com]

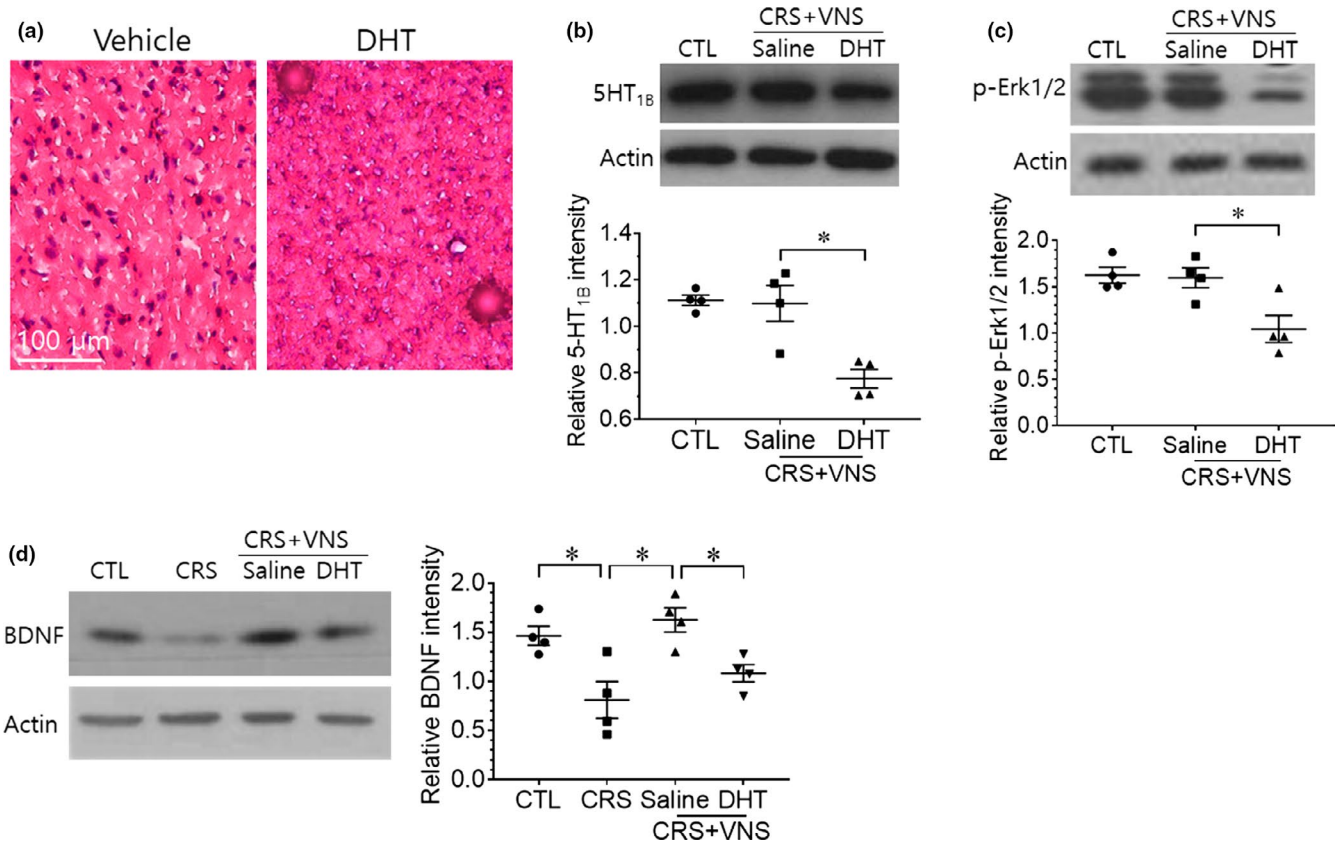

FIGURE 3 Effects of 5,7-DMT injection on hippocampal production of 5-HT$_{1B}$ receptors, phospho-Erk1/2, and BDNF in CRS + VNS animals. (a) Hemotoxylin and eosin (H & E) staining of sagittal brain sections in DRN area. (b–d) Western blot analyses of hippocampal proteins from animal groups as indicated in the figure. In (b) and (d), images in the upper panel are the representatives from four independent experiments, and quantification plots of relative protein band intensity to actin are shown in the lower panel. *$p < 0.05$ (One-way ANOVA). [Colour figure can be viewed at wileyonlinelibrary.com]

we asked whether chronic VNS applied to CRS animals was involved in regulating neuronal responses and further linked to behavioral consequences. Specifically, we examined serotonergic receptors in the hippocampal neurons and found that production of 5-HT$_{1B}$ receptors was reduced after chronic stress but then elevated by VNS. Production of phospho-Erk1/2 and BDNF was similarly regulated by VNS in CRS animals. These changes appeared to relate with antidepression-like responses, thus implying that VNS may be linked to both hippocampal neuronal activation and behavioral effects.

Among seven serotonin receptor families (5-HT$_1$—5-HT$_7$ receptors), 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors in 5-HT$_1$ family have been well characterized as potential mediators regulating depression. Mice deficient in 5-HT$_{1A}$ receptors showed elevated anxiety and antidepressant-like response (Heisler et al., 1998; Ramboz et al., 1998) and developmental expression of 5-HT$_{1A}$ receptors induced to rescue anxiolytic behavior in adult animal (Gross et al., 2002). Like 5-HT$_{1A}$ receptors, 5-HT$_{1B}$ receptors act as both presynaptic autoreceptors and non-serotonergic postsynaptic heteroreceptors, thus function to regulate the release of neurotransmitters in several brain areas (Ruf & Bhagwagar, 2009; Sari, 2004). Genetic polymorphism of human 5-HT$_{1B}$ receptor gene was shown to be associated with major depression (Huang et al., 2003), and activation of 5-HT$_{1B}$ receptors by administration of selective agonist induced antidepressant-like effect in mice (Chenu et al., 2008). Yeast two-hybrid screening identified the interaction of 5-HT$_{1B}$ and 5-HT4 receptors with p11 in several brain areas, and further interaction of p11 with downstream effector molecules was implicated by anti-depressive activity in experimental animals (Svenningsson, Kim, Warner-Schmidt, Oh, & Greengard, 2013; Svenningsson et al., 2006).

In the present study, we found that hippocampal production of 5-HT$_{1B}$ receptors is regulated by chronic VNS in CRS animals. Electrical stimulation of the vagus nerve around the ascending carotid artery activated neurons in the DRN as demonstrated by c-Fos induction. These neurons were positive to 5-HT$_{1A}$ receptors, suggesting that they are autoreceptors that may act to regulate the release of neurotransmitters including serotonin in the axon terminals (Artigas, 2013; Haj-Dahmane, Hamon, & Lanfumey, 1991). Following VNS, serotonergic neurons in the DRN may facilitate signal transmission to several brain areas including

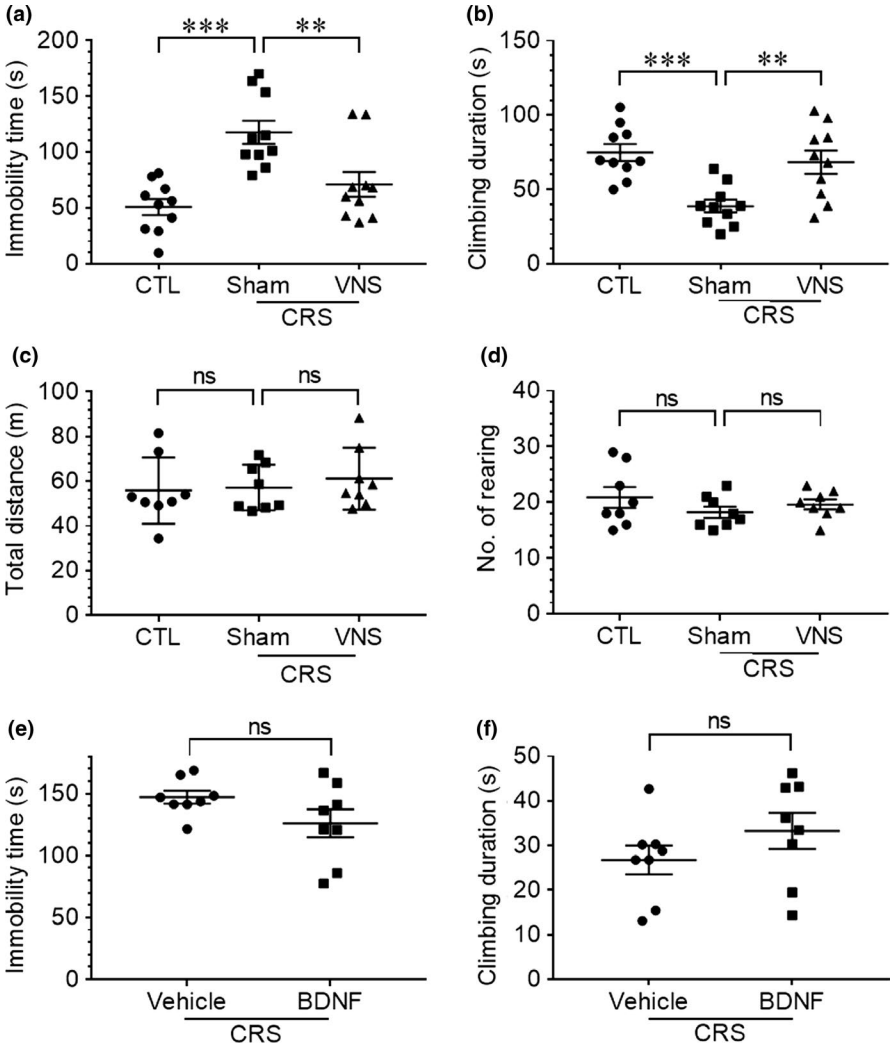

FIGURE 4 Effects of VNS on immobility and climbing scores and spontaneous movement in CRS animals. Rats were subjected to the FST, and durations of immobility (a) and climbing (b) were measured separately for 5 min test period. Scores of total distance of movement (c) and rearing (d) were recorded for 5 min and compared among CTL, CRS + Sham, and CRS + VNS groups of rats. In (e) and (f), CRS rats were injected with BDNF or saline vehicle and subjected to FST to measure the duration of immobility (e) and climbing (f) behaviors. Number of animals per each group in (a) and (b), (c) and (d), and (e) and (f) were 10, 8, and 8, respectively. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ One-way ANOVA in (a-d) and unpaired t test in (e-f). ns, not significant

hippocampus and upregulate the production of heterosynaptic 5-HT_{1B} receptors. Previous histological studies showed that moderate levels of 5-HT_{1B} receptor are expressed in the hippocampal CA1 neurons (Voigt, Laurie, Seeburg, & Bach, 1991) and low levels of expression in the dentate granule cells (Egeland, Warner-Schmidt, Greengard, & Svenningsson, 2011). Here, our data showed that hippocampal levels of 5-HT_{1B} receptors were upregulated by VNS in CRS animals, showing intense signals in the striatum pyramidale and radiatum of CA1 neurons. It was previously reported that the interaction of 5-HT_{1B} receptors with p11 activates annexin A2 and SMARCA3 pathway to induce transcriptional activation of target genes, which is further linked to antidepression-like effects (Svenningsson et al., 2013). Whether VNS-induced activation of 5-HT_{1B} receptors is related to p11-associated antidepressant pathway remains to be explored.

Our data further showed that hippocampal neurons increasing to the production of 5-HT_{1B} receptors by VNS also upregulated phospho-Erk1/2 signals, as has been demonstrated in rat hippocampus (Carreno & Frazer, 2014). Activation of 5-HT1 receptors induces the phosphorylation of Erk2 through the involvement of βγ subunit of G protein (Mendez, Kadia, Somayazula, El-Badawi, & Cowen, 1999; Wirth, Holst, & Ponimaskin, 2017), raising the possibility that increased phosphorylation of Erk1/2 may act as a downstream effector of 5-HT_{1B} receptors in hippocampal neurons of CRS + VNS animals. In addition, previous studies reported that VNS increased ΔFosB immunoreactivity in several brain areas including noraderenergic locus coeruleus and parabrachial nucleus (Furmaga, Sadhu, & Frazer, 2012; Furmaga et al., 2011). Thus, the possibility that neuronal inputs such as noradrenergic activity from locus coeruleus increase Erk1/2 phosphorylation in hippocampal neurons cannot be ruled out (Kong, Ruan, Qian, Liu, & Le, 2010).

Increases in BDNF production in several brain areas including hippocampus are known to be related to antidepressant activity in experimental animals (Nestler et al., 2002; Tsankova et al., 2006). In one study, hippocampal BDNF expression was attenuated after CRS, and then elevated by combined administration of quetiapine and venlafaxine for

antidepression effects (Xu et al., 2006), and in other studies, administration of VNS to normal rats enhanced BDNF expression in the hippocampus (Biggio et al., 2009; Follesa et al., 2007). Here, we demonstrate that the hippocampal production of BDNF is decreased in CRS animals, which is then upregulated by VNS. How would the VNS be involved in regulating the hippocampal production of BDNF in CRS animals? Previous studies showed that, after administration of tricyclic antidepressant imipramine into the mice with chronic social defeat stress, BDNF gene expression was induced by the downregulation of histone deacetylase 5 (HDAC5) and subsequent increases of splice variants of BDNF mRNA through the chromatin remodeling of BDNF gene promoter (Tsankova et al., 2006). In addition to acetylation and methylation, phosphorylation by Erk1/2 can modify histone proteins and epigenetic regulation of target gene expression (Cheung, Allis, & Sassone-Corsi, 2000). Thus, one possible speculation is that VNS may be directly involved in epigenetic regulation of BDNF gene expression in animal models of depression. Alternatively, Erk1/2 activity, which was induced through the activation of 5-HT_{1B} receptors after chronic VNS in CRS animals, may phosphorylate histone 3 and regulate target gene expression including BDNF (Cheung et al., 2000).

Our data show that application of VNS in CRS animals upregulates the production of 5-HT_{1B} receptors in the hippocampal neurons and further activates Erk1/2 and BDNF pathway, as a possible downstream signaling event. Changes in responsiveness of Erk1/2 and BDNF in CRS + VNS animals are well correlated with the improved immobility and climbing scores in the FST. Hippocampal administration of BDNF in CRS animals resulted in decreasing immobility and increasing climbing scores in FST but failed to show statistical significance. It is thus speculated that VNS-induced BDNF activity may be involved in generating antidepression-like behavioral effect in CRS animals, but hippocampal BNDF itself would not be sufficient enough to lead to behavioral consequences. This notion is consistent with a previous report showing that hippocampal injection of BDNF potentiated antidepression-like activity of paroxetine as measured by swimming behavior in FST (Deltheil et al., 2009). VNS-induced serotonergic activity can affect other brain areas besides hippocampus, thus the exploration of the responsiveness of molecular factors including BDNF in other brain areas is equally important as well. In conclusion, our study demonstrates that VNS can be applied successfully to animal models of depression and is useful to investigate molecular factors that are related to behavioral antidepressive activity.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA ACCESSIBILITY

The corresponding author is happy to provide data upon reasonable request.

AUTHOR CONTRIBUTIONS

HCS, BGJ, and KWL designed the study, conducted the experiments, and analyzed data. CYL designed the study and analyzed data. UN designed the study, analyzed data, and wrote the manuscript. All the authors approved the final manuscript.

ORCID

Uk Namgung https://orcid.org/0000-0001-5253-1143

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