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Induction of c-Fos and ΔFosB Immunoreactivity in Rat Brain by Vagal Nerve Stimulation

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Vagus nerve stimulation (VNS) is used as therapy for treatment-resistant depression or epilepsy. This study used immunohistochemistry for biomarkers of short-term (c-Fos) and long-term (ΔFosB) neuronal activation to map regions in brain that are activated by acute (2 h) or chronic (3 weeks) VNS in conscious Sprague–Dawley rats. Electrodes (Cyberonics Inc.) were implanted on the left vagus nerve and 1 week after surgery, stimulation began using parameters employed clinically (one burst of 20 Hz, 250 μs pulse width, 0.25 mA stimulation for 30 s every 5 min). Radio telemetry transmitters were used for monitoring blood pressure, heart rate, activity, and respiratory rate during VNS; neither acute nor chronic VNS significantly affected these parameters. Acute VNS significantly increased c-Fos staining in the nucleus of the solitary tract, paraventricular nucleus of the hypothalamus, parabrachial nucleus, ventral bed nucleus of the stria terminalis, and locus coeruleus but not in the cingulate cortex or dorsal raphe nucleus (DRN). Acute VNS did not affect ΔFosB staining in any region. Chronic VNS significantly increased ΔFosB and c-Fos staining bilaterally in each region affected by acute VNS as well as in the cingulate cortex and DRN. Using these stimulation parameters, VNS was tested for antidepressant-like activity using the forced swim test (FST). Both VNS and desipramine significantly decreased immobility in the FST; whereas desipramine decreased immobility by increasing climbing behavior, VNS did so by increasing swimming behavior. This study, then, identified potential sites in brain where VNS may produce its clinical effects.

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INTRODUCTION

Vagal nerve stimulation (VNS) has been approved by the Food and Drug Administration for treatment of refractory epilepsy (1997) and more recently (2005) for treatment of refractory depression. With respect to efficacy in depression, a 12-week study comparing severely ill patients who received VNS in addition to the medications they had been receiving did not improve significantly more than those who received sham treatment (Rush *et al*, 2005a). However, follow-up of those patients who had received VNS and continued receiving such stimulation for an additional 9 months or switching the sham-treated patients to VNS for 12 months indicated a time-dependent increase in efficacy (Rush *et al*, 2005b). After 12 months of VNS, both response and remission rates were about double those at 3 months. In this cohort, it was found that if patients responded beneficially to VNS after either 3 or 12 months of treatment, then efficacy was maintained for up to 24 months (Sackeim *et al*, 2007).

How VNS produces its beneficial clinical effects is unknown. The vagus is a mixed nerve with 80% of the fibers carrying afferent sensory information to the central nervous system (CNS) (Foley and DuBois, 1937; Nemeroff *et al*, 2006). The cell bodies of the afferent fibers are located in the nodose ganglia and project to the nucleus of the solitary tract (NTS). The NTS then sends direct projections to many regions, including cell bodies for noradrenergic or serotonergic neurons, namely the locus coeruleus (LC) and dorsal raphe nucleus (DRN), respectively (Groves and Brown, 2005; Nemeroff *et al*, 2006), with such neurons thought to be involved in the mechanisms of action of antidepressants. In addition to these direct projections, the NTS sends indirect projections widely throughout the brain (Groves and Brown, 2005; Nemeroff *et al*, 2006). Human imaging studies carried out with VNS also show widespread effects on subcortical and cortical regions (Barnes *et al*, 2003; Henry *et al*, 2004, 1998). Thus, stimulation of the vagus would be expected to cause widespread effects in many areas of brain.

Several studies have examined the ability of VNS to 'activate' brain regions using increases in Fos protein (Curran and Morgan, 1995; Herdegen and Leah, 1998; Senba and Ueyama, 1997) as the measure of activation (Gieroba

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and Blessing, 1994; Naritoku *et al*, 1995; Osharina *et al*, 2006; Rutherfurd *et al*, 1992; Yousfi-Malki and Puizillout, 1994). All these studies measured the ability of acute VNS, for 30–180 min, to increase Fos protein in brain of either rats or rabbits. In all but one study (Gieroba and Blessing, 1994), stimulation was carried out in anesthetized animals. Anesthesia can increase Fos protein throughout brain (Takayama *et al*, 1994), which could mask effects of vagal stimulation. Anesthesia can also change the threshold for activation of different types of fibers in the vagal bundle (Woodbury and Woodbury, 1990). Further, in all but one study (Naritoku *et al*, 1995), stimulation of the vagus was carried out using either electrodes or stimulation parameters that resulted in alterations of peripheral autonomic function, (eg mean arterial pressure, MAP; heart rate, HR; and/or respiratory frequency, RF). Such changes would be expected to produce reflexes that could activate brain regions thereby complicating the interpretation of the results.

In view of these considerations, it seemed worthwhile to reexamine this issue using clinically relevant stimulation parameters in conscious rats. Also, given the long-term nature of VNS, it also appeared useful to examine effects of VNS after more chronic stimulation. Consequently, in nonanesthetized rats we studied the effects of both acute (2 h) or more chronic (3 weeks) VNS using c-Fos or ΔFosB as markers of activation. Fos is an immediate early gene product and has been used to indicate acute activation of cells, usually peaking within 1–3 h of stimulus exposure (Kovacs, 1998). By contrast, FosB and its splice variant ΔFosB show a more delayed activation but persist longer than c-Fos; consequently, they have been suggested to be markers of chronic neuronal activation (Nestler, 2004).

MATERIALS AND METHODS

Animals

Experiments were carried out using adult male Sprague–Dawley rats, 250–350 g (Charles River). Rats were individually housed and maintained in a temperature-controlled environment on a 14 : 10 h light–dark cycle. Rats had *ad libitum* access to food and water. Experimental protocols were approved by the IACUC in accordance with the guidelines of the Public Health Service, American Physiological Society, and Society for Neuroscience.

Vagus nerve electrodes were implanted on the left vagus nerve under aseptic conditions. The surgical procedure was similar to that described by Dorr and Debonnel (2006) except that the anesthetic was 2% isofluorane. Electrodes were connected to a stimulator pack that was sutured in and placed in a subcutaneous pouch created on the back of the rat. Both the electrode and stimulator were supplied by Cyberonics Inc. (Houston, TX). The bipolar stimulating electrode was configured with the cathode as the proximal lead and the anode at the distal lead to preferentially direct action potential propagation toward the CNS by creating anodal block at the distal lead. Rats that received VNS ($n = 15$) were instrumented with an operational stimulator pack that was programmed by a handheld computer. Controls received a dummy simulator pack ($n = 14$) that was the same size and weight (48 mm × 33 mm × 7.1 mm; 16 g).

Telemetry

Some rats were also instrumented with a radio telemetry transmitter (Data Sciences Instr.) so as to monitor systolic and diastolic blood pressure, HR, RF, and activity. Systolic and diastolic blood pressures were used to calculate an MAP that was used for statistical analysis. Physical activity was assessed by measuring changes in the animal's transmitter signal strength. When the animal changed position, the change in transmitter signal strength relative to the reference point was measured as an increase in counts/min. Low counts indicated reduced physical activity in the animal. The activity data provided by this system is strictly a measure of locomotor activity and has been used widely for this purpose (Ansah *et al*, 1996; Howarth *et al*, 2005; Kawashima *et al*, 1996; Meerlo *et al*, 1999; Zhang *et al*, 2004). For the acute study, radio telemetry signals were recorded continuously from each individual at a rate of 64 Hz. Signals were averaged during a 1 h baseline period before stimulation, during the first hour of VNS or sham stimulation, the second hour of VNS or sham stimulation, and for a 30 min poststimulation or sham recovery period. In the chronic study, radio telemetry signals were sampled continuously for 10 s every 10 min, 24 h a day for the duration of the experiment. Signals were recorded for 5 days prior to stimulation and throughout the 3-week stimulation period. The 10 s samples were used to create hourly averages that were further averaged over the 24 h period to generate daily means.

Acute VNS

Seven days after implantation, the vagus nerve was stimulated for 2 h (one burst of 20 Hz, 250 μs pulse width, 0.25 mA output current for 30 s every 5 min) in six rats, whereas five rats served as sham controls. These stimulation parameters are very similar to those used clinically (Rush *et al*, 2005a, b). Thirty minutes after the 2 h vagus nerve or sham stimulation period, rats were anesthetized and perfused intracardially. All rats in this study were instrumented with radio telemetry transmitters.

Chronic VNS Stimulation

Rats ($n = 9$) received continuous VNS for 3 weeks with the same duty cycle and stimulus parameters used in the acute study. Nine rats served as sham controls. Six rats from the VNS group and six rats from the sham group received radio telemetry transmitters. Rats were perfused 30 min after the end of the experiment.

Fos and ΔFosB Immunohistochemistry

All rats were anesthetized with pentobarbital (50 mg/kg i.p.) and perfused with 0.1 M phosphate-buffered saline (PBS) followed by 300–500 ml of 4% paraformadlehyde in PBS. Brains were removed and placed in PBS with 30% sucrose for 3–4 days. Each brain was marked on the left side

(ipsilateral to the VNS implant) and sectioned in a cryostat. Three serial sets of 40 μm coronal sections from each brain were placed in cryoprotectant and stored at −20°C until processed for immunohistochemistry (Cunningham *et al*, 2002).

Separate sets of serial sections were stained for either c-Fos (Rabbit anti-c-Fos Ab5, Calbiochem, San Diego, CA) or FosB (Goat anti-FosB (102; sc-46 g), Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (Howe *et al*, 2004; Ji *et al*, 2005). The primary antibody used in this study does not discriminate between FosB and its splice variant ΔFosB. However, ΔFosB accumulates with chronic stimuli as a result of its long half-life, particularly the 37 kDa isoform (Nestler, 2004), which is the isoform detected by the antibody used. For this reason, we refer to chronic stimulation increasing ΔFosB in this study although a contribution from FosB cannot be excluded. For c-Fos immunohistochemistry, sections were incubated with the primary antibody (1 : 30 000) for 72 h at 4°C. Sections processed for FosB staining were incubated with the primary antibody (1 : 5000) for 72 h at 4°C. The sections were incubated in biotyinlated horse anti-rabbit IgG or horse anti-goat IgG (Vector Laboratories, Berlingame, CA) diluted 1 : 200 in PBS for 2 h at room temperature. Sections were reacted with an avidin–peroxidase conjugate (Vectastain ABC Kit; Vector Laboratories) and PBS containing 0.04% 3,3′-diaminobenzidine hydrochloride and 0.04% nickel ammonium sulfate for 10 to 11 min. Brain stem sections were then processed for dopamine-β-hydroxylase (DBH) immunofluorescence (Curtis *et al*, 1999; Grindstaff *et al*, 2000; Ji *et al*, 2005). Sections were mounted on gelatin-coated slides, which were air-dried for 1–2 days, and coverslipped with Permount.

Forebrain regions included in the analysis were the paraventricular nucleus of the hypothalamus, supraoptic nucleus, amygdala, bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the thalamus, vertical limb of the diagonal band of Broca, cingulate cortex, and insular cortex. The following brain stem regions were also analyzed: the dorsal raphe, LC, NTS, area postrema, caudal ventrolateral medulla, rostral ventrolateral medulla, and parabrachial nucleus. DBH immunofluorescence was used anatomically to define catecholamine-containing regions and ensure that sections were obtained from the same rostral-caudal plane for each set of sections from each rat. Tissue sections containing regions of interest were inspected using an Olympus microscope (IX 50) equipped for epifluorescence. Digital images were acquired using a Spot camera (SPOT RT Slider, Diagnostic Instruments, Sterling Heights, MI) connected to a Pentium computer running Spot Imaging software (v. 3.24). Some images were adjusted for brightness and contrast in order to standardize them for analysis. Regions of interest were identified using the rat brain stereotaxic atlas of Paxinos and Watson (1986). Three to six images were taken from each region for each animal bilaterally as previously described (Cunningham *et al*, 2002, 2007; Howe *et al*, 2004; Ji *et al*, 2005). The number of c-Fos or ΔFosB positive cells were recorded for each image and averaged for each animal. Counts were generated by observers who were blind to the treatment condition associated with the images (Cunningham *et al*, 2002, 2007; Howe *et al*, 2004; Ji *et al*, 2005).

Forced Swim Test

After 1 week of recovery, rats were trained and tested in the forced swim test (FST) using the method of Lucki (1997), with minor modifications. Training and test sessions were recorded by a video camera positioned above the swimming tank. On the training day, the rats were placed into a cylindrical tank (60 × 30 cm) containing 25°C water at a depth of 35 cm for 15 min. The first 5 min of the 15 min session were recorded on videotape. Water was changed between subjects. After 24 h, the test session was carried out in the same manner for 5 min, and recorded in its entirety.

One-half hour after the rats were removed from the water on the training day, they were injected either with saline or desipramine (DMI), or the vagal nerve stimulators were turned on (using the handheld computer). Rats received three sessions of VNS, using the stimulation parameters described above, for 2 h each, starting 30 min after the training session and then at 6.5 and 2.5 h before the test session. This sequence mimics the way drugs are usually given in the FST (Cryan *et al*, 2002a). Sham VNS controls were implanted with a 'dummy' simulator and electrodes placed around the left vagus nerve as described above. DMI, used at a dose of 15 mg/kg, s.c., was injected 23.5, 2.5, and 0.5 h before the test session. A control group of rats received injections of 0.9% NaCl at the same intervals. The videotaped behavior of the rats was scored using a time-sampling technique to rate the predominant behavior over a 5 s interval as described by Lucki (1997). Immobility was defined as floating or no active movements made other than that necessary to keep the nose above the water. Swimming consisted of active motions throughout the swim tank and crossing into another quadrant, but not having the forepaws break the surface of the water. Climbing was defined as upward-directed movements of the forepaws against the wall and/or having the forepaws break or churn the surface of the water in vigorous swimming.

Statistical Analysis

Telemetry data from the acute and chronic studies were analyzed using separate two-way mixed effect analyses of variance (ANOVAs) and Newman–Keuls *t*-tests were used for *post hoc* analysis (SigmaStat, v. 2.03, Systat Software Inc., Point Richmond, CA). Data from the cell counts were analyzed by one-way ANOVA with Newman–Keuls *t*-test for *post hoc* analysis. Statistical analyses of the FST results were performed by Student's *t*-tests. All values are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

RESULTS

Telemetry

In the acute study, baseline values for rats receiving VNS were slightly higher for average MAP and somewhat lower for HR and RF compared with values for the sham group, but these differences were not statistically significant. Acute VNS did not significantly affect MAP, HR, RF, or activity as compared with the prestimulation baseline values or values for the sham group (Figure 1). Activity, as measured by the

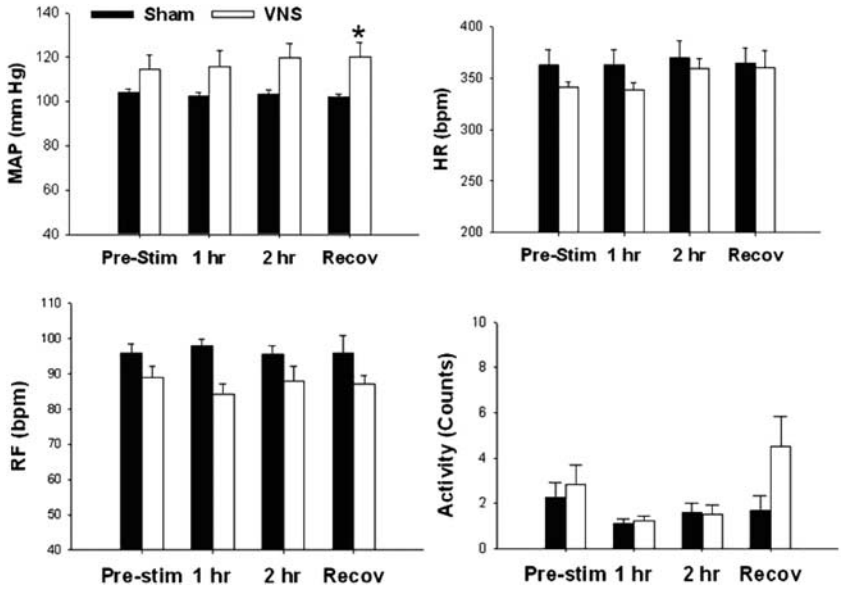

Figure 1 Effects of acute left vagus nerve stimulation (VNS) on mean arterial pressure (MAP), heart rate (HR), respiratory frequency (RF), and locomotor activity in rats receiving vagus nerve stimulation for 2 h (VNS, $n=6$) or sham controls ($n=5$). Each variable was analyzed using separate two-way mixed effects ANOVA. *Significantly different from sham, $P<0.05$ (Student's Newman–Keuls *t*-test).

telemetry signal, was not significantly different between the two groups before or during VNS; there was a significant main effect for time due to an increase in activity during the recovery period (Figure 1).

Throughout the duration of the chronic study, the VNS group showed a trend toward a higher RF as compared to the sham group, but this trend was not statistically significant (Group $F(1,9)=4.42$, $P=0.065$; data not shown). HR decreased significantly during the stimulation period, but this decrease occurred in the sham rats as well as those that received VNS (Time $F(25,225)=13.7$, $P<0.01$). Activity in both groups also significantly varied across time during the experiment (Time $F(25,225)=4.2$, $P<0.01$). However, the differences were not statistically significant between the VNS and control groups either during the 5-day baseline period or during the 3 weeks of VNS for any of the measured variables. Thus, neither acute nor chronic VNS altered any of the parameters measured any differently than that seen in rats receiving sham VNS.

Fos and ΔFosB Immunohistochemistry

Several brain regions involved in reflex cardiovascular control, such as the caudal ventrolateral medulla, rostral ventrolateral medulla, and dorsal motor nucleus of the vagus were not affected by either acute or chronic VNS (Figure 2). These results are consistent with the telemetry data because no cardiovascular or respiratory effects were associated with either acute or chronic VNS. Other areas not affected significantly by either acute or chronic VNS, using these stimulation parameters, included the amygdala, supraoptic nucleus of the hypothalamus, diagonal band of Broca, and insular cortex.

In contrast, several regions of the CNS were affected by both acute and chronic VNS. The most commonly observed pattern of staining was best illustrated by the NTS. Acute VNS significantly increased Fos staining bilaterally in the NTS with no effect on ΔFosB staining (Figures 2 and 3). After chronic VNS, both Fos and ΔFosB staining were increased bilaterally in the NTS (Figures 2 and 3). This pattern of staining was also observed in the LC and the ventral portion of the BNST as well as the parabrachial nucleus and the paraventricular nucleus of the hypothalamus (Figure 4).

Two regions showed a different pattern of staining associated with VNS. Both the DRN and the anterior cingulate cortex showed increased ΔFosB staining following chronic VNS, but Fos staining was not affected by either acute or chronic VNS (Figures 5 and 6). In the paraventricular nucleus of the thalamus, Fos staining was not affected by acute or chronic VNS. However, this region demonstrated high basal levels of ΔFosB staining that significantly decreased in association with chronic VNS (Chronic Sham 47.3 ± 4.6; Chronic VNS 25.5 ± 2.8, $P<0.01$, Newman–Keuls test). This was the only region in which a significant decrease in either Fos or ΔFosB staining occurred.

Forced Swim Test

As acute VNS elevated c-Fos in several brain areas, it was of interest to determine whether such stimulation caused a behavioral effect in the rats. The FST is a widely used behavioral test used to detect potential antidepressant-like activity (Cryan *et al*, 2005a; Porsolt *et al*, 1977). Consequently, we compared the effect of VNS in the FST with that produced by a standard antidepressant, DMI. As expected, DMI reduced immobility significantly in the FST and, also as expected (Cryan *et al*, 2005b), did so by an increase in climbing rather than swimming behavior (Figure 7). VNS also reduced immobility when administered similarly to DMI, (ie three times for 2 h each session between the

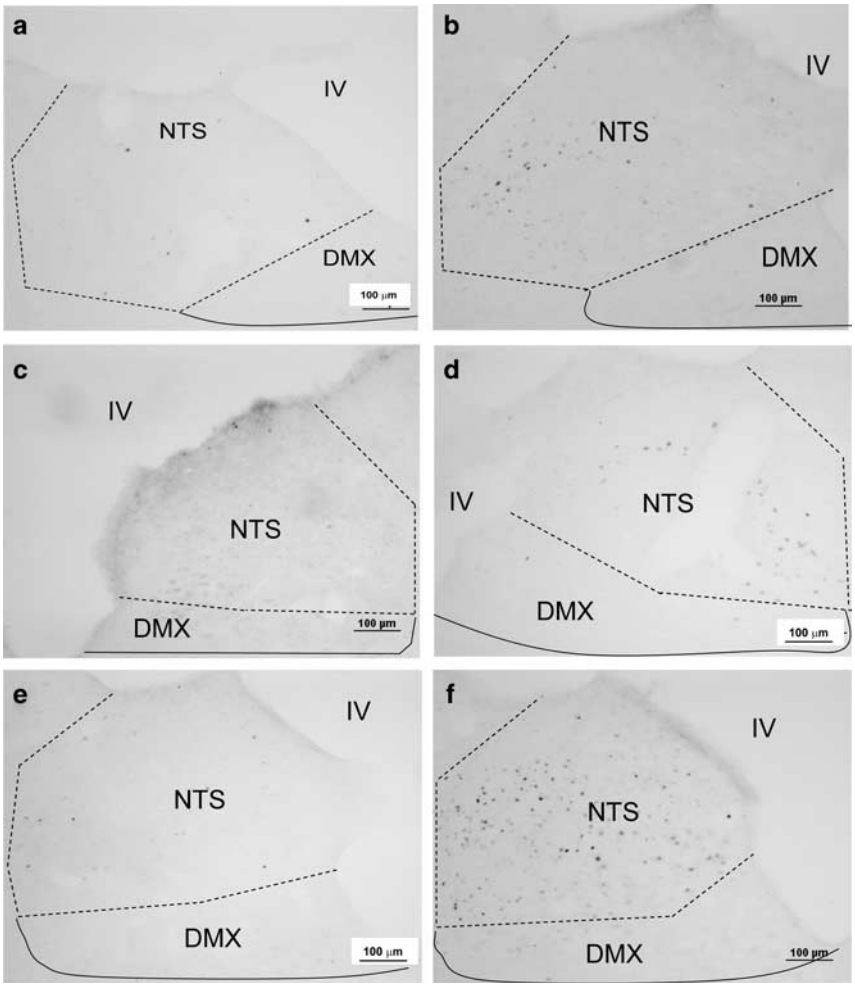

Figure 2 Representative digital images of c-Fos and ΔFosB staining in the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMX) following either acute (2 h) or chronic (3 weeks) VNS. (a) Fos staining for an acute sham animal. (b) Fos staining following acute VNS. (c) Fos staining from a chronic sham rat. (d) Fos staining after chronic VNS. (e) ΔFosB staining in a chronic sham rat. (f) ΔFosB staining after chronic VNS. Note that following VNS the nuclear staining that is characteristic of Fos and ΔFosB staining is located primarily in the NTS with little or no staining in the DMX.

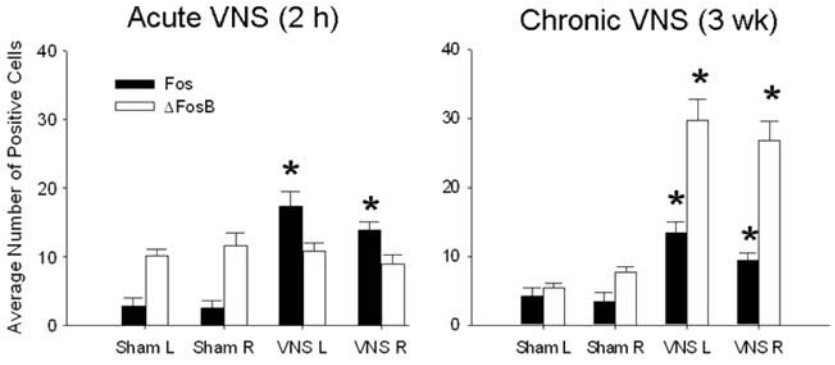

Figure 3 Summary data for the effects of acute (left) and chronic (right) vagus nerve stimulation (VNS) on Fos and ΔFosB staining in the NTS. L and R on the x axis indicate the left and right NTS from Sham and VNS-treated rats. In each group the electrode was implanted on the left vagus nerve. Acute VNS significantly increased Fos staining in both the left and right NTS without affecting ΔFosB staining. Chronic VNS was associated with significant bilateral increases in both Fos and ΔFosB. *Significantly different from sham, $P<0.05$ (Student's Newman–Keuls *t*-test).

training and test sessions). In contrast to DMI, though, the decrease in immobility resulted from an increase in swimming rather than from climbing behavior (Figure 7). During the training session, no significant differences in immobility were observed between the rats designated to receive either saline or DMI (33 ± 2 *vs* 35 ± 2, respectively) or those that received sham VNS compared with those that received actual stimulation (23 ± 2 *vs* 21 ± 2, respectively).

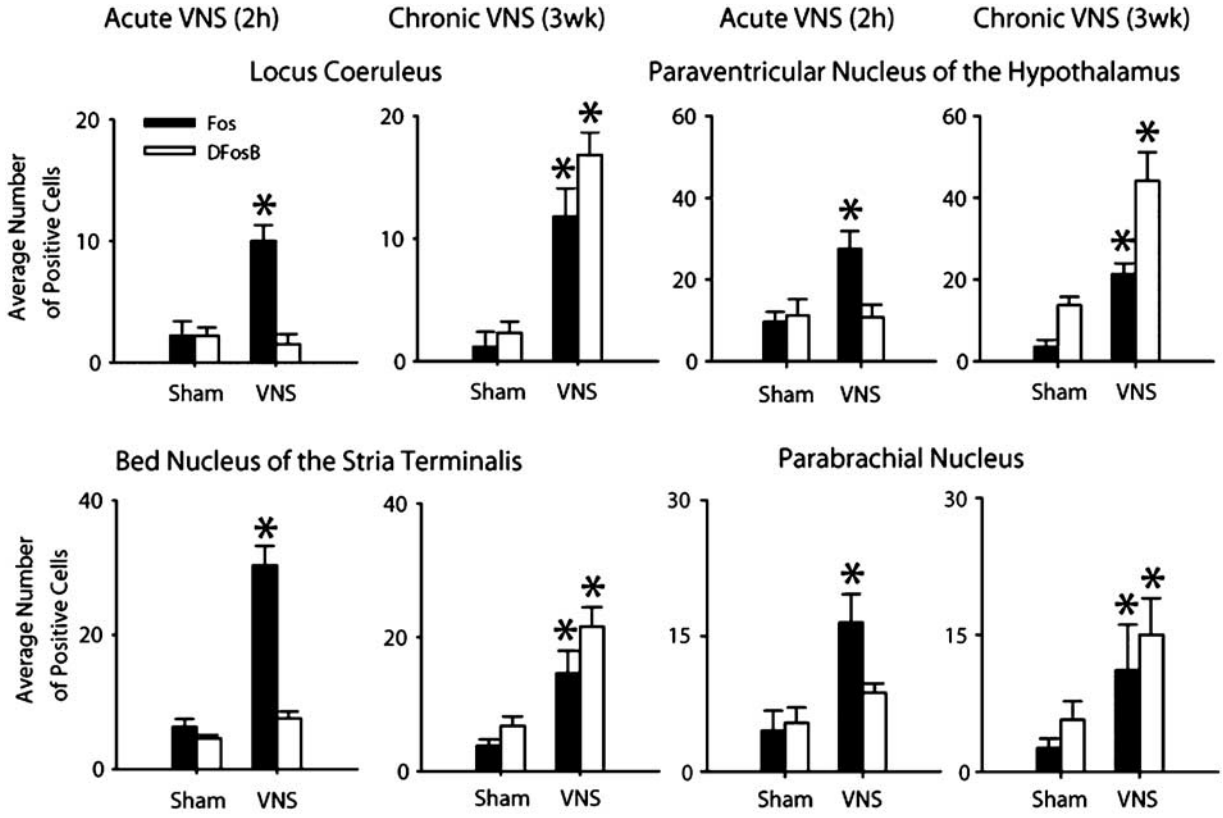

Figure 4 Effects of acute and chronic vagus nerve stimulation (VNS) on Fos and ΔFosB staining in the locus coeruleus, ventral bed nucleus of the stria terminalis, parabrachial nucleus, and paraventricular nucleus of the hypothalamus. Data from the ipsilateral side are presented in the graphs, although VNS produced significant increases bilaterally in both regions as was observed in the NTS. *Significantly different from sham, $P<0.05$ (Student's Newman–Keuls *t*-test).

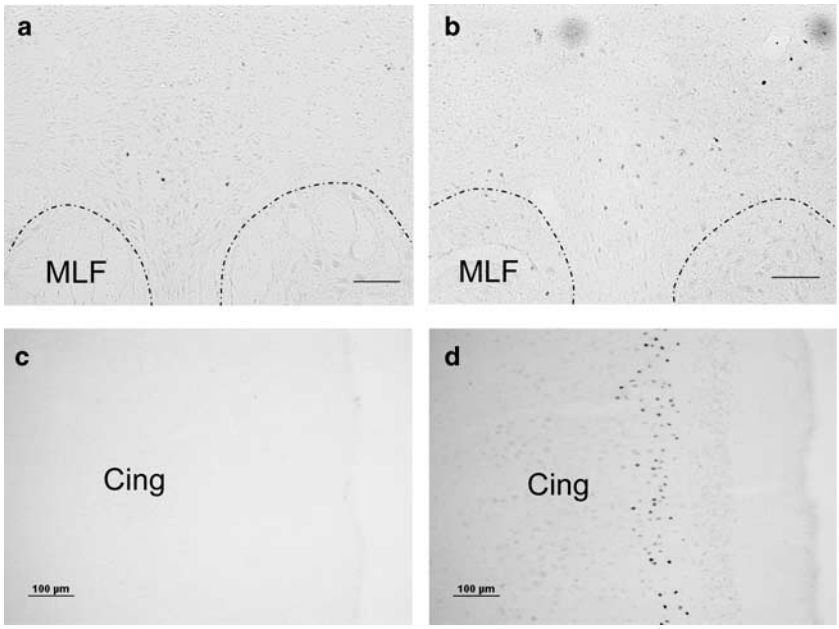

Figure 5 Representative digital images illustrating the effects of chronic VNS on ΔFosB staining in the dorsal raphe nucleus (DRN, top row) and anterior cingulate cortex (Cing, bottom row). (a and c) ΔFosB staining in a chronic sham rat. (b and d) ΔFosB staining following chronic VNS. MLF, medial longitudinal fasiculas.

Telemetry data from the acute VNS study indicated that VNS does not significantly affect general activity (Figure 1). Similarly, the telemetry data from the chronic VNS study also indicates that the first 6 h of VNS did not significantly affect general activity (6 h prestimulation: Sham 2.2 ± 0.6, VNS 2.5 ± 0.3; 6 h VNS: Sham 1.8 ± 0.3, VNS 2.2 ± 0.3;

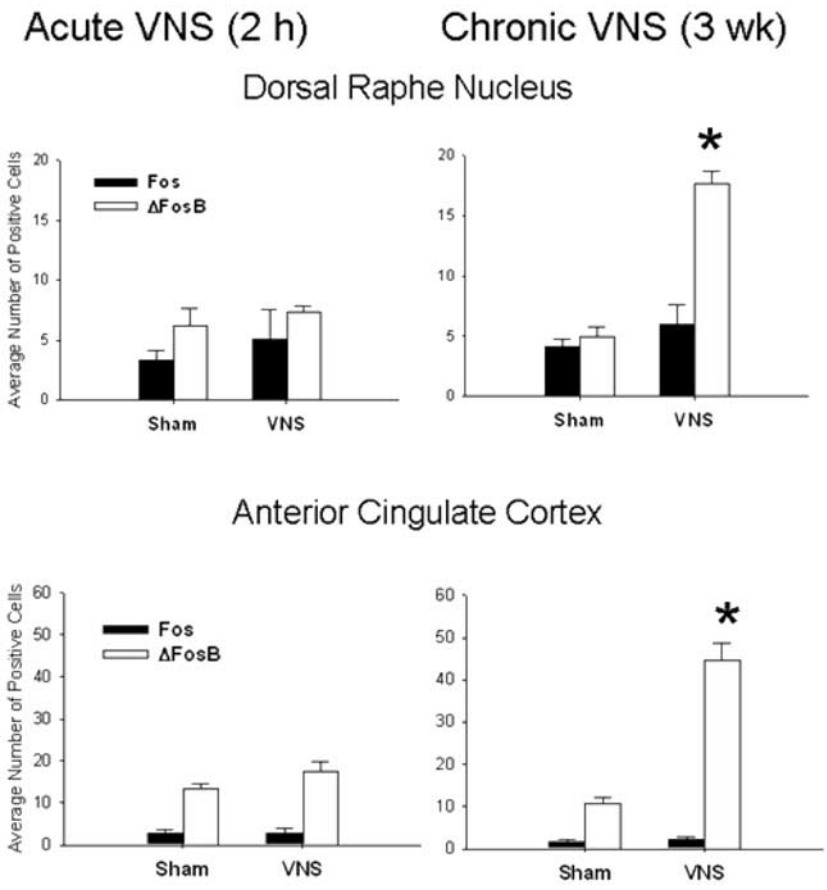

Figure 6 Effects of acute and chronic vagus nerve stimulation (VNS) on Fos and ΔFosB staining in the dorsal raphe nucleus (top) and the anterior cingulate cortex (bottom). In both regions, acute VNS was not associated with significant changes in either Fos or ΔFosB staining. Chronic VNS significantly increased ΔFosB staining with no effect on Fos. *Significantly different from sham, $P<0.05$ (Student's Newman–Keuls *t*-test).

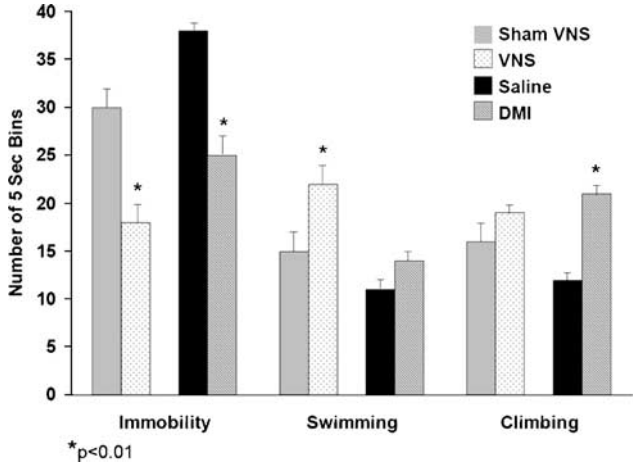

Figure 7 Effects of desipramine (DMI) treatment and vagus nerve stimulation (VNS) on immobility, swimming, and climbing activity in the forced swim test. *Significantly different from control value, $P<0.01$ (Student's *t*-test).

$F(1,10)=1.34$, $P>0.1$). It is unlikely, therefore, that the decrease in immobility caused by VNS results from a general increase in locomotor activity.

DISCUSSION

Acute unilateral VNS in conscious rats, using clinically relevant stimulation parameters, increased staining for c-Fos bilaterally in the NTS and many, but not all, regions that receive its projections. Chronic VNS for 3 weeks also elevated c-Fos in the same areas as acute VNS. Whereas acute VNS never increased staining for ΔFosB, chronic VNS did so routinely. Even in the DRN and cingulate cortex where neither acute nor chronic VNS caused any increase in staining for c-Fos, staining for ΔFosB was increased significantly after chronic stimulation. Further, activation of brain using these stimulation parameters was sufficient to cause a behavioral effect, namely reduced immobility in the FST.

The electrode configuration used in this study was designed to create an anodal block at the distal end of the nerve (personal communication, William Buras, Cyberonics Inc.) so as to decrease the activation of vagal efferents. The anodal block is the result of current flowing from the anode to the cathode causing hyperpolarization at the anode and depolarization at the cathode. This hyperpolarization blocks the propagation of action potentials at the distal end of the electrode limiting the activation of vagal efferents. The anodal block seems to be effective with this stimulation

because neither acute nor chronic stimulation caused changes in HR, MAP, or RF, even though central effects were produced by VNS. Consistent with this lack of peripheral autonomic effects was the absence of increased staining for either c-Fos or ΔFosB in areas involved in cardiovascular or respiratory reflexes, such as the ventrolateral medulla or dorsal motor nucleus of the vagus (Dampney and Horiuchi, 2003; Guyenet, 2006). These findings support the likelihood that the VNS-induced increases centrally in either c-Fos or ΔFosB result directly from VNS and not indirectly from reflex activation of peripheral afferent inputs secondary to changes in MAP, HR, or RF.

The absence of cardiovascular or respiratory effects using this electrode configuration and stimulation parameters is in contradistinction to previous studies on the effects of acute VNS on c-Fos in either the rabbit or the rat. In previously reported experiments, VNS produced marked effects on HR, MAP, and/or RF (Gieroba and Blessing, 1994; Naritoku *et al*, 1995; Osharina *et al*, 2006; Rutherfurd *et al*, 1992). Also, most of these studies were carried out using anesthetized animals and stimulation protocols with higher frequencies and duty cycles than the protocols used clinically and in the present study. Anesthesia also lowers activation of C fibers by VNS (Woodbury and Woodbury, 1990). Consequently, the effects reported in these earlier studies using anesthetized preparations may be distinct from those in unanesthetized animals. Such results, then, are unlikely to have much relevance for what occurs clinically when VNS is used in epilepsy or depression.

In the present study, stimulation of the vagus nerve for 2 h significantly increased staining for c-Fos in the NTS, parabrachial nucleus, LC, ventral BST, and paraventricular nucleus of the hypothalamus. Because the NTS is the primary termination site for vagal afferents (Kalia and Sullivan, 1982), the increase in staining for c-Fos is not unexpected. Also, the caudal two-thirds of the NTS is innervated by both the ipsilateral and the contralateral vagus nerves (Norgren, 1978; Ter Horst *et al*, 1989), so, bilateral activation of c-Fos in the NTS by stimulation of the left vagus was also anticipated. The NTS has a diverse network of afferent projections and it has been described as having three types or sets of afferent projections (Saper, 1995). First, the NTS has descending projections to autonomic preganglionic neurons. Second, it has projections to areas within the medullary reticular formation, such as the caudal and rostral ventrolateral medulla that are involved in cardiovascular and respiratory reflex control (Dampney, 1994; Guyenet, 2006). Third is a set of ascending projections from the NTS to midbrain, hypothalamic, and cortical regions that are involved in central autonomic control (Groves and Brown, 2005; Nemeroff *et al*, 2006). Included in this ascending system are direct projections to many regions, including cell bodies for noradrenergic or serotonergic neurons, ie the LC and DRN, respectively, with such neurons thought to be involved in the mechanisms of action of antidepressants (Lenox and Frazer, 2002). The LC has also been implicated in the anticonvulsant effects of VNS as well (Krahl *et al*, 1998). The NTS also sends indirect projections widely throughout the brain that originate from its direct projections (Castle *et al*, 2005; Groves and Brown, 2005; Nemeroff *et al*, 2006). For example, a recent study using transneuronal tract tracing demonstrated that visceral afferents from the NTS may influence the ventral hippocampus through a circuit that includes the LC, thalamus, and BST (Castle *et al*, 2005).

Human imaging studies of VNS also show widespread effects on subcortical and cortical regions (Barnes *et al*, 2003; Henry *et al*, 1998, 2004). Thus, stimulation of the vagus would be expected to cause widespread effects in many areas of brain including regions involved in affect and cognition. However, areas such as the amygdala, diagonal band of Broca, supraoptic nucleus, ventral hippocampus, DRN, and ventrolateral medulla did not show increases in c-Fos staining even though they also receive either direct projections from the NTS and/or innervation from areas to which the NTS projects (Castle *et al*, 2005; Dampney, 1994; Nemeroff *et al*, 2006). At least as measured by c-Fos, the stimulation parameters used in this study are not causing sufficient stimulation of the NTS to result in increased c-Fos staining in all of its projection areas. It is also possible that in some of these regions, such as the amygdala or supraoptic nucleus, VNS may be associated with inhibitory effects that would not be reflected by changes in c-Fos staining. Negative results must be interpreted with caution because previous studies have demonstrated that some populations of neurons require sustained stimulation in order to produce c-Fos (Dampney and Horiuchi, 2003; Dragunow and Faull, 1989).

One previous study measured c-Fos in rats after acute VNS using the Cyberonics stimulator (Naritoku *et al*, 1995). Anesthetized rats received VNS for 3 h using the same 10% duty cycle that we used although the frequency (30 Hz), current (1 mA), and stimulus pulse duration (0.5 ms) were greater. Cardiovascular or respiratory parameters were not measured. In contrast to our results, Naritoku *et al* (1995) found high staining for c-Fos in a nucleus of the amygdala and cingulate cortex as well as the dorsal motor nucleus of the vagus and the NTS. Similar to our results, they found activation in the LC but not in the DRN. Given the difference in stimulation parameters and the use of anesthetized rats, some differences in results are to be expected.

Of course, VNS is administered chronically when used clinically. Consequently, it was of interest to compare the pattern of activation in areas of brain after acute (2 h) or chronic (3 weeks) VNS. ΔFosB was measured to reflect chronic activation (Cunningham *et al*, 2007; Gottlieb *et al*, 2006; Herdegen and Leah, 1998; Ji *et al*, 2005; Nestler, 2004). There are four major protein members of the Fos family: c-Fos, FosB, Fra-1, and Fra-2 (Herdegen and Leah, 1998; Kovacs, 1998; Nestler, 2004). In general, maximal levels of c-Fos protein occur within 1–3 h of stimulus exposure and then disappear in 4–6 h, whereas FosB and its splice variant, ΔFosB, show a more delayed activation but persist longer (Chen *et al*, 1997). The accumulation of ΔFosB with chronic stimuli is a result of its long half-life, particularly the 37 kDa isoform (Nestler, 2004), which is the isoform detected along with FosB by the antibody used in this study.

In all areas in which acute VNS increased staining for c-Fos, chronic VNS did as well, although the increases were generally somewhat less than those seen with acute stimulation. As anticipated, chronic VNS elevated ΔFosB

and it did so in all areas where either acute or chronic VNS increased staining for c-Fos. Increased ΔFosB staining associated with chronic VNS is likely due to the accumulation of this protein resulting from intermittent activation in these regions over the 3 weeks of stimulation. Increased c-Fos staining after chronic VNS also could be related to the intermittent nature of the stimulation protocol (ie rats only receive stimulation for 10% of the time). However, increased c-Fos staining associated with chronic experimental manipulations such as water deprivation and hypertension has been previously reported (Cunningham *et al*, 2007; Ji *et al*, 2005; Miyata *et al*, 2001).

Chronic VNS also increased ΔFosB in the DRN and the cingulate cortex where increases in c-Fos staining had not been seen with either acute or chronic stimulation. These results indicate that VNS at these stimulation parameters produces acute activation of the LC but only a more delayed activation of the DRN and a cortical area, the cingulate . The activation of the cingulate by chronic VNS contrasts with the results of imaging studies in patients in which decreased subgenual cingulate (Cg25) activity was found in association with clinical response to various types of antidepressant treatments (Goldapple *et al*, 2004; Mayberg *et al*, 2000; Nobler *et al*, 2001). Also chronic deep brain stimulation of the white matter tracts adjacent to the subgenual cingulate produced a marked beneficial effect in four of six patients with treatment refractory depression (Mayberg *et al*, 2005). The cingulate cortex would appear to be an important area to study in future research on antidepressant treatments.

The temporal pattern of activation of the LC and DRN is of interest given the importance of norepinephrine and serotonin in the mechanism of action of antidepressants (Lenox and Frazer, 2002) and the continuing improvement in depression produced over time by VNS (Groves *et al*, 2005; Rush *et al*, 2005b). The time course of activation in the present study is consistent with the results of Dorr and Debonnel (2006) who measured the ability of VNS to increase the firing rate of LC or DRN neurons. These investigators also used stimulators supplied by Cyberonics Inc. and the same stimulation parameters used by us. In conscious rats, stimulation of the vagus for as little as 1 h produced increases in the firing rate of LC neurons, with the firing rate further increasing linearly for up to 90 days. By contrast, no increases in the firing rate of neurons in the DRN were seen for up to 3 days of stimulation; however, their firing rate increased significantly after 14 days of VNS with the firing rates increasing even further after 90 days of stimulation. Groves *et al* (2005) also reported recently that acute stimulation of the vagus nerve in anesthetized rats increased the firing rate of LC neurons. Acute activation of the LC by VNS is consistent with reports that the LC seems to be necessary for its acute antiseizure activity in an animal model (Krahl *et al*, 1998) and that acute VNS increases extracellular norepinephrine (Roosevelt *et al*, 2006).

In view of our results with VNS, of relevance are studies that have measured mRNA for *c-fos* or c-Fos protein after antidepressant treatments. Essentially all studies were conducted after acute (30 min–2 h) administration of antidepressants. A reasonably consistent observation is that administration of pharmacologically diverse antidepressants (SSRIs, selective NRIs, MAOIs, mirtazapine) increase *c-fos* or c-Fos in the central nucleus of the amygdala (CeA) and BNST (Beck, 1995; Duncan *et al*, 1996; Fraga *et al*, 2005; Miyata *et al*, 2005; Morelli *et al*, 1999; Slattery *et al*, 2005; Sumner *et al*, 2004; Thomsen and Helboe, 2003). This increase was also seen in female rats treated acutely with fluoxetine (Torres *et al*, 1998). Most studies report no increase in the DRN, although Fraga *et al* (2005) did find an increase after administration of fluoxetine. When increases in the LC have been reported, it has been upon administration of the SSRI fluoxetine (Fraga *et al*, 2005; Slattery *et al*, 2005; Sumner *et al*, 2004) but, with one exception (Beck, 1995), not with other types of antidepressants. In general, increases have not been reported in the hippocampus and many other regions of brain and effects in cortical areas have been quite inconsistent. Acute electroconvulsive shock (ECS) also increases c-Fos in cortex and hippocampus (Cole *et al*, 1990; Hope *et al*, 1994; Morinobu *et al*, 1997; Winston *et al*, 1990). Interestingly, chronic treatment of rats with either paroxetine (Muigg *et al*, 2007) or citalopram (Kuipers *et al*, 2006) does not increase c-Fos in any brain region examined, including the CeA. We have found only one study of chronic antidepressant treatment on ΔFosB. In that study, ECS increased ΔFosB in cortex and hippocampus after 2 days and the effect became maximal after 4 days (Hope *et al*, 1994).

Not surprisingly, comparing such results with the acute VNS results in the present study reveals both similarities and differences. Perhaps most striking is that VNS did not increase c-Fos in any amygdaloid nuclei, although it did in the BNST. Also, VNS increased c-Fos in the LC, whereas this was either not seen or seen inconsistently with antidepressants other than fluoxetine. Similar to the result with antidepressants, acute VNS did not increase c-Fos in the DRN. Also, we found chronic VNS to increase c-Fos (as well as ΔFosB) in areas of brain, whereas this has not yet been observed with chronic administration of SSRIs. In view of these effects of VNS, it would be useful to investigate whether antidepressants produced effects in areas not examined previously, such as the NTS or parabrachial nucleus. Whether such differences in effects between VNS and antidepressants contributes to the utility of VNS in treatment-resistant depression remains to be determined.

Given the limited activation of brain caused by these stimulation parameters, it was of interest to see if they would produce a behavioral effect. To explore this, the FST was used. This test was originally described by Porsolt *et al* (1977) to detect antidepressant-like activity in rats and has become one of the more widely, if not the most widely, used procedure for detecting antidepressant-like activity in rodents (Cryan *et al*, 2005a, b). The antidepressants are often given three times between the first and second exposure to the swim tank. Consistent with many other reports (Cryan *et al*, 2002b), when DMI, the noradrenergic reuptake inhibitor, was administered in this way it caused a decrease in immobility that was due to an increase in climbing, but not swimming, behavior. There are substantial data that antidepressants that act selectively through norepinephrine increase climbing but not swimming behavior (see Cryan *et al*, 2002b), whereas serotonergic antidepressants reduce immobility by increasing swimming

activity rather than climbing activity (Dziedzicka-Wasylewska *et al*, 2006; Page *et al*, 1999; Xu *et al*, 2000). VNS, when administered for thee sessions of 2 h each between the two swim sessions, produced a comparable reduction in immobility to DMI. These results are consistent with those of Krahl *et al* (2004) who found that 30 min of continuous VNS each day for 4 days reduced immobility in the FST. Thus, stimulation with these clinically relevant parameters can produce an antidepressant-like effect in rats. However, the decrease in immobility caused by VNS was due to an increase in swimming rather than in climbing activity. Given this difference, it is reasonable to speculate that the effect of VNS in the FST is more likely due to a serotonergic rather than to a noradrenergic mechanism. However, the results with c-Fos show no effect of VNS in the DRN after 2 h of stimulation even though an effect was found in the LC. In the forced swim experiment, VNS was given three times for 2 h each session over 23 h. Future experiments should explore whether such a paradigm causes an increase in either c-Fos or ΔFosB in the raphe and whether lesions of serotonergic or noradrenergic neurons influence the effects of VNS in the FST.

In conclusion, the present study identified potential sites in brain where VNS may produce its clinically significant effects. A limited number of sites showed activation, as measured by c-Fos, after acute stimulation with chronic stimulation showing activation of the same sites as acute stimulation plus two more, namely the DRN and the anterior cingulate cortex. Future research will be necessary to determine the functional implications of activation of these sites.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that this research was funded in part by an extramural research grant from Cyberonics Inc. the manufacturer of the VNS device used in this study.

The authors declare that over the past 3 years JTC, SWM, and AF have received fiscal compensation as consultants for Cyberonics Inc. In addition, AF has served as a consultant or on advisory boards for Lilly, Lundbeck, Sepracor, and Wyeth. In addition, GG declares that except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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