COMPARISON OF ΔFOSB IMMUNOREACTIVITY INDUCED BY VAGAL NERVE STIMULATION WITH THAT CAUSED BY PHARMACOLOGICALLY DIVERSE ANTIDEPRESSANTS

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VNS, sertraline & DMI induce ΔFosB activation in rat brain

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List of abbreviations:
DMI: desipramine
SERT: sertraline
VNS: vagal nerve stimulation
TRD: treatment-resistant depression

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Vagal nerve stimulation (VNS) has been approved for treatment refractory depression. Yet there have been few if any studies directly comparing effects produced by VNS in animals with those caused by antidepressants, particularly using clinically relevant stimulation parameters in non-anesthetized animals. In this study, ΔFosB immunohistochemistry was used to evaluate different brain regions activated by chronic administration of VNS. Effects of VNS were compared with those caused by sertraline (SERT) or desipramine (DMI). Double-labeling of ΔFosB and serotonin was used to determine whether serotonergic neurons in the dorsal raphe nucleus (DRN) were activated by chronic VNS. VNS significantly increased ΔFosB staining in the nucleus tractus solitarius (NTS), parabrachial nucleus (PBN), locus coeruleus (LC) and DRN, as well as in many cortical and limbic areas of brain including those involved in mood and cognition. Most, but not all, of these effects were seen also upon repeated treatments of rats with sertraline or DMI. Some areas where VNS increased ΔFosB (e.g. the NTS, PBN, LC and peripeduncular nucleus) were not affected significantly by either drug. Sertraline was similar to VNS in causing an increase in the DRN whereas DMI did not. Double-labeling of the DRN with ΔFosB and an antibody for serotonin revealed that only a small percentage of ΔFosB staining in the DRN co-localized with serotonergic neurons. Effects of VNS were somewhat more widespread than those of caused by the antidepressants. The increases in ΔFosB produced by VNS were either equivalent to and/or more robust than those seen with antidepressants.
INTRODUCTION

Vagal nerve stimulation (VNS) has been approved by the Food and Drug Administration for treatment-resistant epilepsy (1997) and for treatment-resistant depression (TRD) (2005). Clinical data show that both response (27-53%) and remission (15-33%) rates over 12 months were significantly higher in patients who received VNS treatment in addition to medications compared to what was reported in another study with similar patients who received only medications (12% response and 4% remission (see Dunner et al., 2006)) and such improvement continues for 24 months (see Rosa and Lisanby, 2012).

In spite of these promising clinical results, there have been relatively few preclinical studies of the effects produced by VNS in animals, particularly using clinically relevant stimulation parameters in non-anesthetized animals. Most of the previous studies examined only acute effects of VNS in anesthetized animals (Rutherfurd et al., 1992, Yousfi-Malki and Puizillout, 1994). Anesthesia can change the threshold for activation of different types of fibers in the vagal bundle (Woodbury and Woodbury, 1990). In most of the earlier studies also, stimulation was carried out using stimulation parameters that resulted in changes in peripheral autonomic function, which would produce reflexes that could activate brain regions so as to complicate the interpretation of the results.

We found previously (Furmaga et al., 2011) that VNS given as it was in this study and for a similar time period caused anxiolytic-like and antidepressant-like effects. It was of interest then to examine what regions of brain were activated when VNS caused behavioral effects.

Immunohistochemistry of c-Fos, an immediate early gene, has become the most widely used functional anatomical mapping tool to identify activation of cells (Kovacs, 1998). There are four major members of the Fos family: c-Fos, FosB, Fra-1 and Fra-2. FosB has a splice
variant termed ΔFosB. These proteins respond to stimuli with different time courses. In general, maximal levels of c-Fos protein occur within one to three hours of stimulus exposure and disappear in four to six hours whereas ΔFosB shows a more delayed activation but persists longer (McClung et al., 2004). Hence, c-Fos has been suggested to be an indicator of acute neuronal activation whereas ΔFosB may reflect longer-term neuroadaptations (McClung et al., 2004).

Several studies have examined mRNA for c-fos or c-Fos protein after antidepressant treatments, with the majority studying acute effects (Beck, 1995, Fraga et al., 2005, Slattery et al., 2005). Data from these studies have been inconsistent with respect to activation in the dorsal raphe nucleus (DRN), locus coeruleus (LC), hippocampus and cortical areas of brain. In addition, chronic treatment of rats with either paroxetine (Muigg et al., 2007) or citalopram (Kuipers et al., 2006) did not increase c-Fos in any brain region examined. We measured c-Fos and ΔFosB previously and found that chronic (3 weeks) administration of VNS in conscious Sprague-Dawley rats significantly increased ΔFosB staining in several areas of brain (Cunningham et al., 2008). Because of this and the aforementioned literature, it was of interest to compare the activation patterns of chronic VNS in many more regions of brain than those studied previously with those produced by chronic treatment with antidepressants, primarily focusing on ΔFosB. The antidepressants selected for study, sertraline (SERT) and desipramine (DMI), target serotonin and norepinephrine neurons, respectively. This is the first study to compare directly such activation patterns after chronic treatment with VNS and antidepressants in non-anesthetized rats. In light of its effectiveness in TRD, we speculated that VNS might cause a broader pattern of activation than that seen with antidepressants or the extent of activation in certain areas would be greater than that seen with the drugs.
MATERIALS AND METHODS

Experiments were carried out using adult male Sprague-Dawley rats, 250-350g (Harlan, Houston, TX). Rats were group 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 Cunningham et al. (2008) except that the anesthetic was a combination of 75 mg/kg ketamine and 0.5 mg/kg medetomidine. Briefly, the coil electrode was placed around the left cervical vagus nerve and carotid sinus ventral to the carotid bifurcation. The bipolar stimulating electrode was configured with the cathode at the proximal lead and the anode at the distal lead to preferentially direct action potential propagation toward the central nervous system by creating an anodal block at the distal lead. The electrodes were connected to a stimulator pack (Cyberonics, Inc., Houston TX) that was sutured in place in a subcutaneous pouch created on the back of the rat. Rats that received VNS were instrumented with an operational stimulator pack that was programmed by a handheld computer. Controls received a dummy simulator pack that was the same size and weight (48 mm x 33 mm x 7.1 mm; 16 g). Beginning 7 days after surgery, rats received continuous VNS treatment for 14 days. The stimulation paradigm consisted of one burst of 20 Hz, 250 μsec pulse width, 250 μA current for 30 sec every 5 min for 2 weeks. These stimulation parameters are very similar to those used initially in clinical studies (e.g. Rush et al., 2005) although parameters may change if patients do not respond. Also, we found previously that this stimulation protocol does not cause changes in blood pressure, heart rate, respiratory frequency or locomotor activity in comparison...
with that measured in rats receiving “dummy” stimulation (Cunningham et al., 2008). However, raising the stimulation current to 500 µA did cause autonomic effects (unpublished data). Thirty min after the end of continuous VNS for 14 days, rats were perfused and the brains were removed for subsequent analysis of ΔFosB.

**Implantation of Osmotic Minipumps:** One day prior to surgery, osmotic minipumps delivering 5 µl/h (Model 2ML2, DURECT Corporation, Cupertino, CA) were filled with drug or vehicle, filtered through 0.9-µm nitrocellulose filters (Millipore, Bedford, MA) using a sterile technique under an air-filtered hood. Drug solution concentrations were determined based on the mean weight of the rats over the 14 days of treatment. Doses were 7.5 mg/kg/day of sertraline or 10 mg/kg/day of desipramine as these have been shown previously to produce serum concentrations of drug in the therapeutic range (Benmansour et al., 1999). Specific surgical details are given in Furmaga et al. (2011). Rats were perfused at the end of the experiment with the minipumps still in place and the brains removed for subsequent immunohistochemical analysis.

**Immunohistochemistry of ΔFosB:** The staining procedure was similar to that described by Cunningham et al. (2008). Briefly, rats were anesthetized with a cocktail of 75 mg/kg ketamine and 0.5 mg/kg medetomidine and perfused with 0.1 M phosphate-buffered saline (PBS) followed by 500 ml of 4% paraformaldehyde in PBS. Brains were removed and placed in PBS with 30% sucrose for 4 days. Three serial sets of 40 μm coronal sections were cut in a cryostat and placed in cryoprotectant and stored at -20 °C until processed for ΔFosB immunohistochemistry.

Sets of serial sections were stained for FosB (Goat anti-FosB (102; sc-46g), Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibody used in this study does not discriminate between FosB and its splice variant ΔFosB. However, due to the fact that ΔFosB accumulates with chronic stimulation as a result of its long half-life, particularly the 37kDa isoform
(McClung et al., 2004), the antibody chosen for this study can specifically detect this isoform. For this reason, we refer to chronic stimulation increasing ΔFosB levels although a contribution from FosB cannot be excluded. To assess ΔFosB, sections were incubated with FosB antibody (1:5000) for 72 h at 4 °C. The sections were then incubated with Alexa Fluor 488 donkey anti-goat IgG (1:1000, Molecular Probes, Carlsbad, CA). Some sets of sections were double-labeled for ΔFosB and serotonin (1:300, Abcam, Cambridge, MA). Sections were then incubated with Alexa Fluor 488 donkey anti-goat IgG (1:1000) for FosB and Alexa Fluor 546 donkey anti-mouse IgG (1:1000) for serotonin at room temperature for 4 h. The sections were washed in PBS and mounted on gelatin-coated slides and coverslipped using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). No immunoreactivity was detected with controls that were incubated with either primary or secondary antibody alone.

The number of ΔFosB positive cells per section in selected brain regions was quantified by observers blind to the experimental conditions as previously described (Cunningham et al., 2008). More areas were analyzed than in our previous study and included the nucleus tractus solitaries (NTS), LC, DRN, parabrachial nucleus (PBN), basolateral (BLA) and central (CeA) nuclei of the amygdala, hippocampus, frontal cortex (Fr), cingulate cortex (Cg), nucleus accumbens (NAc), striatum, bed nucleus stria terminalis (BNST), substantia nigra (SN) or ventral tegmental area (VTA), and peripeduncular area (PP). The areas were defined based on the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 1986). Areas in which cell counts were quantified are shown with red boxes over representative atlas schematics (Figure 1). Sections were examined by fluorescent microscopy. For quantitative analysis, at least four representative sections from each brain region were imaged. Digital images were collected using
an Olympus BX40 microscope equipped with a DP72 Olympus camera connected to a Pentium computer running imaging software.

**Single-labeling in situ hybridization for c-fos mRNA:** The labeling procedure was similar to that described by Liu et al. (2007). Antisense $^{35}$S-labeled cRNA probes for rat c-Fos were generated with $^{35}$S-UTP and $^{35}$S-CTP using standard transcription system. Brain sections were mounted on polylysine-coated slides and fixed in 4% paraformaldehyde for 1 hr and rinsed in 2x SSC (300 mM NaCl, 30 mM Na citrate, pH 7.2). Brain sections were acetylated in 0.1 M triethanolamine, pH 8.0, with 0.25% acetic anhydride (for 10 min) and dehydrated through a graded series of alcohol (50-100%) and subsequently air-dried. $^{35}$S-labeled cRNA probes were diluted to $3 \times 10^4$ cpm/µl in 50% hybridization buffer (50% formamide, 10% dextran sulfate, 3x SSC, 50 mM sodium phosphate buffer, pH 7.4, 1x Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM DTT). Brain sections were hybridized with 70 µl of the diluted probes and placed in plastic trays moistened with 50% formamide at 55 °C overnight. The following day, coverslips were lifted with 2x SSC, and slides were rinsed three times for 5 min each in 2x SSC, and incubated in RNAse A buffer containing 200 µg/ml RNAse A for 1 hr at 37 °C followed by a series of washes of increasing stringency (2x, 1x, 0.5x, and 0.1x SSC, for 5 min each at room temperature). The sections were then placed in 0.1x SSC at 65 °C for 1 hr, rinsed in distilled water, and dehydrated through a graded series of alcohol. Brain sections were exposed to x-ray film (BioMax MR, Eastman Kodak, Rochester, NY) for 7 days.

Levels of mRNA for c-fos were evaluated by analyzing film autoradiography. Films were visualized under a CCD camera (Model XC-77; Sony, Tokyo, Japan), and brain section images were captured and analyzed with the NIH ImageJ image analysis system. Signals were expressed as optical density levels above threshold. The threshold level was defined as 3.5 SDs above the
mean optical density of a region. Results were expressed as integrated optical density, which is
the product of the signal intensity and number of pixels above the threshold within the defined
brain region. Equivalent planes of coronal brain sections through the DRN were ensured for
analysis between animals.

Statistical Analysis: Mean of cell counts from multiple sections was calculated for each rat and
then the mean of all rats in a treatment group was determined. Data were analyzed by one-way
ANOVA followed by Student’s Newman-Keuls post-hoc tests. To enable direct comparisons
between the effects of VNS and the antidepressants, a one-way ANOVA was carried out for all
groups for each area of brain. P<0.05 was considered statistically significant. All values are
presented as mean +/- SEM.
RESULTS

Administration of VNS for 14 days to conscious Sprague-Dawley rats significantly increased ΔFosB staining in the NTS [F(4,19) = 195.6, p < 0.0001] and regions that receive both direct and indirect projections from it such as the PBN [F(4,19) = 69.87, p < 0.0001], LC [F(4,19) = 49.58, p < 0.0001], and DRN [F(4,19) = 32.56, p < 0.0001] (Figure 2). Because the NTS and PBN are not traditional targets of monoamine-based antidepressants, it was not unexpected that they were not affected significantly by either chronic administration of sertraline or DMI (Figure 2). Sertraline significantly increased ΔFosB staining in the DRN, although the effect was not as robust as VNS. The patterns of activation were similar between VNS and sertraline within the DRN, with ΔFosB staining mainly localized to the lateral wings with little activation in the ventromedial subnucleus. By contrast, noradrenergic neurons in the LC were not activated either by DMI or sertraline (Figure 2).

As essentially all the cells in the LC are noradrenergic (Swanson, 1976), it can be assumed that it is noradrenergic cells that are activated by VNS in the LC. However, only about 40% of the cells in the DRN are serotonergic (Nanopoulos et al., 1982). Consequently, double-labeling immunohistochemistry was carried out in the DRN to see if the cells activated, i.e., exhibiting an increase in ΔFosB, were serotonergic. To do this, the cells in the DRN were labeled with an antibody for serotonin. As shown in Figure 3, chronic VNS significantly increased ΔFosB staining in the DRN but only a very small percentage of cells labeled with ΔFosB co-localized with serotonergic cells.

As dopaminergic cell bodies are not traditional targets for antidepressant drugs, it was of interest to see if VNS would cause activation in the SN or VTA. Neither VNS nor the drugs did this (data not shown). Interestingly, on the sections containing the SN and VTA, it was observed
that VNS produced staining in the peripeduncular nucleus \( [F(4,19) = 44.65, p < 0.0001] \) but it was subsequently found that the drug treatments did not do this (Figure 4).

VNS induced widespread increases in \( \Delta FosB \) in many cortical and limbic areas of brain including regions involved in mood and cognition. In all forebrain areas in which chronic sertraline or desipramine increased staining for \( \Delta FosB \), VNS did as well. In general, the increases produced by VNS were either equivalent to (Figure 5) or more robust (Figure 6) than those seen with the antidepressants.

A comparison of the areas activated by chronic treatment with VNS, sertraline, or DMI is presented in Table 1. VNS caused widespread activation with only four areas not showing activation, among 15 examined, namely the CeA, SN, VTA and striatum. The two drug treatments did not activate these areas either (Table 1). Neither sertraline nor DMI elevated \( \Delta FosB \) in the NTS, PB, LC and PP. Whereas sertraline elevated \( \Delta FosB \) in the DRN, desipramine did not.

As previously stated, it is widely accepted that maximal levels of c-Fos protein occur within one to three hours of stimulus exposure and disappear in four to six hours whereas \( \Delta FosB \) shows a more delayed activation but persists longer. Hence, increases in c-Fos have been suggested to be an indicator of acute neuronal activation while \( \Delta FosB \) reflects chronic activation (McClung et al., 2004). Interestingly, we observed increases in c-Fos after chronic VNS or antidepressant drug administration in the same brain regions as seen with \( \Delta FosB \) (data not shown). Because of this somewhat surprising result, quantitative analysis of mRNA levels for \( c-fos \) using in situ hybridization was carried out in rats given VNS. Expression of \( c-fos \) increased in the DRN with VNS treatment, with the increase present throughout the nucleus (Figure 7).
This result implies that the increases in c-Fos measured by immunohistochemistry reflected increases in c-fos.
**DISCUSSION**

VNS significantly increased ΔFosB staining in the NTS and regions that receive either direct and/or indirect projections from it such as the PBN, LC and DRN (Peyron et al., 1996, Berthoud and Neuhuber, 2000). Additionally, VNS induced widespread increases in ΔFosB in many cortical and limbic areas of brain including regions involved in mood and cognition. Most, but not all, of these effects were seen also upon repeated treatments of rats with sertraline or DMI. Neither drug treatment caused an increase in ΔFosB in the LC whereas sertraline was similar to VNS in causing an increase in the DRN. Also, neither drug treatment increased ΔFosB in the peripeduncular nucleus whereas VNS did. In general, the increases produced by VNS were either equivalent to or more robust than those caused with antidepressants.

There is an anatomical rationale for VNS having effects on many cortical and limbic areas of brain involved in mood and cognition. About 80% of vagal fibers carry afferent sensory information to the central nervous system (Foley and DuBois, 1937), with the initial projection area in brain being the NTS (Kalia and Sullivan, 1982). One set of projections from the NTS contains ascending projections from the NTS to the midbrain, hypothalamic, and cortical regions involved in central autonomic control; included in this ascending system are direct/and or indirect projections to the LC and DRN (Peyron et al., 1996, Van Bockstaele et al., 1999, Berthoud and Neuhuber, 2000) containing cell bodies for noradrenergic or serotonergic neurons, respectively and it is well established that such neurons are targets for antidepressants. The NTS also sends projections throughout the brain involving areas thought to be involved in mood and emotion (Berthoud and Neuhuber, 2000)

The stimulation parameters used here are similar to those used initially in clinical studies (e.g. Rush et al., 2005). Also, when VNS was given to rats using these stimulation parameters for
similar time periods as in the current study, both anxiolytic-like and antidepressant-like effects were observed (Furmaga et al., 2011). Consequently, effects seen in this study are likely occurring in rats when VNS is effective behaviorally. If ΔFosB, then, can be used as an index of chronic cellular activation, then some of the brain regions demonstrated to be activated in this study may be involved in the behavioral effects of VNS.

In agreement with our previous study (Cunningham et al., 2008), repeated VNS administration significantly increased ΔFosB staining in the NTS, PBN, LC, and DRN. Moreover, the present study revealed that VNS induced widespread ΔFosB activation in many cortical and limbic areas such as the frontal cortex, cingulate cortex, hippocampus, BNST, and NAc. Results of imaging studies in patients with epilepsy or depression who are treated with VNS also show widespread effects on subcortical and cortical regions, with short term VNS producing increases in blood flow in the hypothalamus, thalamus and insular cortex but decreases in the hippocampus and posterior cingulate gyrus (Chae et al., 2003). Chronic VNS produced both increased (Kosel et al., 2011) and decreased (Nahas et al., 2007) changes in blood flow in cortical regions although subcortical regions were activated (Henry et al., 2004). Inconsistent results were also obtained in the amygdala (Zobel et al., 2005, Conway et al., 2006). VNS causes acute limbic hyper-perfusion and chronic thalamic hypo-perfusion in patients with refractory epilepsy and these changes correlate with clinical efficacy (Vonck et al., 2008).

Data from previous studies that examined mRNA for c-fos or c-Fos protein after acute antidepressant administration have been inconsistent with respect to the LC, DRN and many cortical areas. Somewhat surprisingly, administration of fluoxetine (Fraga et al., 2005, Slattery et al., 2005) but not other types of antidepressants, with one exception (Kovacs, 1998), was found to increase c-Fos in the LC. In general, increases have not been reported in the DRN although
Fraga et al. (2005) found an increase after acute administration of fluoxetine. Most studies show increases in c-fos or c-Fos in the CeA and BNST after acute administration of different classes of antidepressants (Beck, 1995, Fraga et al., 2005, Slattery et al., 2005, Bechtholt et al., 2008). Chronic treatment of rats with either paroxetine (Muigg et al., 2007) or citalopram (Kuipers et al., 2006) did not increase c-Fos in any brain region examined, including the CeA. In contrast, Bechtholt et al. (2008) showed that chronic fluoxetine increased c-Fos in multiple brain areas including the BNST, cingulate cortex, anterior NAc and hippocampus and chronic mirtazapine treatment increased c-Fos in the CeA and dentate gyrus (Gerrits et al., 2006).

There are very few studies that examine the effects of antidepressant drug treatments on ΔFosB. Recently, Vialou et al. (2010) showed that chronic fluoxetine treatment produced an accumulation of ΔFosB in the NAc shell. In addition, they show that viral-mediated over-expression of ΔFosB in the rat NAc produced a significant antidepressant-like effect in the forced swim test.

Comparing such results with those seen after chronic sertraline and DMI in the present study reveals both similarities and differences. This is likely due to the fact that most of the previous work involved acute antidepressant treatment and measured c-Fos whereas we report on ΔFosB. However, although the data were not shown, we measured c-Fos as well and found comparable results with it as were found with ΔFosB. Interestingly, we found that sertraline increased c-Fos staining in the DRN whereas this was not seen with or seen inconsistently with acute antidepressant treatments other than fluoxetine (Fraga et al., 2005). Even though ΔFosB staining increased in the DRN, this was not primarily in serotonergic soma (see Figure 4). In agreement with most previous studies, we found that neither drug treatment caused an increase in ΔFosB in the LC. This is not surprising as activation of α2-autoreceptors in the LC persists in
restraining norepinephrine neurotransmission in the face of tonically elevated basal
norepinephrine levels following chronic reuptake blockade (Garcia et al., 2004). The increase in
\(\Delta FosB\) staining in the LC following VNS stimulation is likely a consequence of efferents from
the NTS, the initial projection area of vagal afferents in brain, innervating noradrenergic
dendrites of LC neurons with synaptic contacts characteristic of both excitatory- and inhibitory-
type transmitters (Van Bockstaele et al., 1999).

Similar to the results found with chronic treatment of rats with paroxetine (Muigg et al.,
2007) and citalopram (Kuipers et al., 2006), none of the treatments in this study increased \(\Delta FosB\)
in the CeA. Vialou et al. (2010) hypothesized that that \(\Delta FosB\) induction in NAc is required for
the antidepressant action of fluoxetine. Whether this is true of sertraline and DMI remains to be
seen although our data show that they, as well as VNS, also increase \(\Delta FosB\) in the NAc.

Chronic VNS and antidepressants increased \(\Delta FosB\) in many cortical areas including the
frontal cortex, cingulate cortex, and NAc. As mentioned, antidepressant drug-induced increases
in these areas have not consistently been observed by others. To the best of our knowledge,
though, we are the first to examine this upon chronic administration of antidepressants
administered using osmotic minipumps so as to obtain steady-state plasma concentrations of
drugs in the therapeutic range. VNS increased \(\Delta FosB\) in the BLA although it did not in the CeA.
The absence of an effect in the CeA is different from that reported by others studying effects of
antidepressants, as mentioned above. However, we also did not see any effect of antidepressants
on \(\Delta FosB\) in the CeA. This difference could be due to the anesthetic used in the previous studies.
It is well known that c-Fos expression can be greatly affected by anesthesia. For example,
barbiturates may interfere with c-Fos expression, whereas other anesthetics such as urethane and
chloralose cause a high level of baseline expression (Miura et al., 1994, Dampney et al., 1995, Rocha and Herbert, 1997).

Little if any overlap was found between the cells showing ΔFosB and those staining for 5-HT in the DRN. This is unexpected because Dorr and Debonnel (2006) showed that repeated administration of VNS to non-anesthetized rats, using stimulation parameters identical to ours, raised the firing rate of noradrenergic neurons in the LC and serotonergic neurons in the DRN. This discrepancy could be due to limitations of using Fos proteins as markers for neuronal activation. Neurons may differ in their capacity to produce Fos; the time-course of Fos induction and decay varies with different inducing stimuli; and some brain regions do not express Fos regardless of stimuli (Dampney and Horiuchi, 2003). Herdegen et al. (1991) reported that the onset of Fos production in somatic motor neurons is considerably delayed (by several hours) compared with most other neurons (typically 30-60 min). The temporal pattern of activation is of great significance given that Dorr and Debonnel (2006) showed that stimulation of the vagus for as little as one hour produced increases in the firing rate of noradrenergic neurons whereas it took 14 days for the firing rate of serotonergic neurons to increase. Moreover, Dragunow et al. (1989) reported that high-frequency stimulation protocols that produced good long-term potentiation do not lead to c-Fos induction. Hence, the absence of ΔFosB in serotonergic neurons in the DRN following chronic VNS does not necessarily mean that they were not activated, especially in light of the data of Dorr and Debonnel (2006).

As shown in Figure 4, the majority of VNS-induced ΔFosB expression in the DRN was observed its lateral margins and comprised an area corresponding to the ventrolateral periaqueductal gray (vLPAG). This distinctive pattern of ΔFosB induction is interesting since the vLPAG has been characterized previously as an important neural substrate for passive coping.
responses (Bandler and Shipley, 1994). Berton et al. (2007) show that the strongest ΔFosB induction in the vLPAG was observed in animals that were most resilient to behavioral despair. They hypothesized that expression of ΔFosB is part of an adaptive mechanism that promotes resilience to stress by inhibiting the stress-induced activation of substance P neurotransmission to the forebrain. This is in good agreement with our behavioral data where we showed that repeated administration of VNS produces anxiolytic-like and antidepressant-like effects in the novelty suppressed feeding test and the forced swim test, respectively (Furmaga et al., 2011).

The in situ hybridization signal for c-fos occurs throughout the DRN whereas ΔFosB immunohistochemistry was localized primarily to the lateral wings. The most likely reason for this discrepancy is that the increase in these two immediate early genes is occurring in different cells, or at least the increase in ΔFosB is occurring in only a subset of cells in which c-fos is elevated. As mentioned previously, these proteins respond to stimuli with different time courses with c-Fos increasing more rapidly than ΔFosB (McClung et al., 2004). Consistent with this, we found previously that 2 hr of VNS increased c-Fos in many brain areas whereas ΔFosB was not increased in any region at this time (Cunningham et al., 2008).

In conclusion, the present study identified potential sites in brain activated by VNS. In general, the effects of VNS were somewhat more widespread than those of caused by the antidepressants and in some areas, the extent of activation was greater with VNS than with the drugs. Whether such differences in effects between VNS and antidepressants contributes to the utility of VNS in treatment-resistant depression remains to be determined.
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Authorship Contribution

Participated in research design: Furmaga and Frazer

Conducted experiments: Furmaga and Sadhu

Contributed new reagents or analytic tools: Furmaga and Sadhu

Performed data analysis: Furmaga and Frazer

Wrote or contributed to the writing of the manuscript: Furmaga, Sadhu and Frazer
REFERENCES


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Legends for Figures

Figure 1: Representative of atlas schematics of areas in which ΔFosB staining (shown with red boxes) was quantified following either chronic VNS, sertraline (7.5 mg/kg/day, i.p) or desipramine (10 mg/kg/day, i.p) treatments. (A) Hippocampus from Begma - 2.40mm to Bregma - 3.60mm. (B) Raphe nucleus from Bregma – 7.32mm to Bregma – 8.28 mm. (C) Nucleus tractus solitarius from Bregma – 13.68 to Bregma – 14.28.

Figure 2: Effects of chronic VNS, sertraline (SERT, 7.5 mg/kg/day, i.p) or desipramine (DMI, 10 mg/kg/day, i.p) on ΔFosB staining in the nucleus tractus solitarius (NTS), locus coeruleus (LC), parabrachial nucleus (PBN) and dorsal raphe nucleus (DRN). *Significantly different from vehicle or sham control, P < 0.05. #Significantly different from SERT, P < 0.05 (One-way ANOVA, Newman-Keuls post-hoc test).

Figure 3: Representative digital images of ΔFosB and 5-HT double staining in the dorsal raphe nucleus in a chronic VNS-treated rat. (A) Representative of an atlas schematic that contains all subnuclei of the DRN (B) ΔFosB staining; (C) 5-HT staining; (D) overlay of ΔFosB and 5-HT staining (E) inset at 40x magnification.

Figure 4: Representative digital images of ΔFosB staining in the peripeduncular nucleus at (A, B) 10x magnification and (C, D) 20x magnification. (A, C) Chronic sham-treated rat; (B, D) Chronic VNS-treated rat; (E) Effects of chronic VNS, sertraline (SERT, 7.5 mg/kg/day, i.p) or desipramine (DMI, 10 mg/kg/day, i.p) on ΔFosB staining in the peripeduncular nucleus. *Significantly different from vehicle or sham control, P < 0.05 (One-way ANOVA, Newman-Keuls post-hoc test).

Figure 5: Effects of chronic VNS, sertraline (SERT, 7.5 mg/kg/day, i.p) or desipramine (DMI, 10 mg/kg/day, i.p) on ΔFosB staining in the frontal cortex [Fr; F(4,19) = 85.95, p < 0.0001], cingulate cortex [Cg; F(4,19) = 80.38, p < 0.0001], hippocampus [Hip; F(4,19) = 8.821, p < 0.001] and basolateral nucleus of the amygdala [BLA; F(4,19) = 16.70, p < 0.0001]. *Significantly different from vehicle or sham control, P < 0.05 (One-way ANOVA, Newman-Keuls post-hoc test).

Figure 6: Effects of chronic VNS, sertraline (SERT, 7.5 mg/kg/day, i.p) or desipramine (DMI, 10 mg/kg/day, i.p) on ΔFosB staining in the nucleus accumbens [NAC; F(4,19) = 82.95, p < 0.0001] and bed nucleus stria terminalis [BNST; F(4,19) = 75.08, p < 0.0001]. *Significantly different from vehicle or sham control, P < 0.05. #Significantly different from SERT or DMI, P < 0.05 (One-way ANOVA, Newman-Keuls post-hoc test).

Figure 7: Effects of VNS on c-fos mRNA levels in the dorsal raphe nucleus (DRN). *Significantly different from vehicle or sham control, P < 0.05 (Student’s t-test).
**Tables**

**Table 1:** Summary data for the effects of chronic vagal nerve stimulation (VNS), sertraline (SERT) or desipramine (DMI) on ΔFosB staining in the rat brain. (+): significant increases in ΔFosB staining; (0): no significant changes.

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<th>LC</th>
<th>PBN</th>
<th>DRN</th>
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NTS: nucleus tractus solitarius; LC: locus coeruleus; PBN: parabrachial nucleus; DRN: dorsal raphe nucleus; Hip: hippocampus; BLA: basolateral amygdala; CeA: central amygdala; NAc: nucleus accumbens; ST: striatum; Fr: frontal cortex; Cg: cingulate cortex; VTA: ventral tegmental area; SN: substantia nigra; PP: peripeduncular nucleus
Figure 4

A

B

C

D

E

Peripeduncular Nucleus

Average Number of Positive Cells

* 30
  25
  20
  15
  10
  5
  0

Vehicle  SERT  DMI  Sham  VNS
Figure 5

Frontal Cortex

Cingulate Cortex

Hippocampus

Basolateral Amygdala

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<tr>
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<th>Sham</th>
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* indicates statistical significance.
Figure 6

![Bar charts showing data for Nucleus Accumbens and Bed Nucleus Stria Terminalis.](chart.png)
Figure 7

![Image of a brain slice comparison between Sham and VNS conditions with a bar graph showing nCi/mg values]