Activation of signaling pathways downstream of the brain-derived neurotrophic factor receptor, TrkB, in the rat brain by vagal nerve stimulation and antidepressant drugs

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Abstract

Vagal nerve stimulation (VNS) has been approved for treatment resistant depression (TRD) by the Food and Drug Administration (FDA) since 2005. However, the cellular and molecular targets responsible for its effects are still not characterized. Previously, chronic administration of VNS to rats was found to phosphorylate tyrosine 515 on TrkB, the neurotrophin receptor, whereas traditional antidepressants did not do this. In the present study, Western blot analysis was used to characterize activation due to phosphorylation in the hippocampus of downstream pathways linked to specific key tyrosine residues on TrkB (namely Y816 and Y515) after either acute or chronic administration of VNS and traditional antidepressant drugs. Chronic administration of VNS caused phosphorylation of effectors linked to Y 515; namely Akt, ERK and p70S6 kinase, but this was not produced by either desipramine or sertraline. All the treatments, when given chronically, caused phosphorylation of the transcription factor, CREB. Acute administration of all the treatments also caused phosphorylation of PLCγ1, but this was not maintained with chronic treatment. Further research is required to determine what role, if any, activation of down-stream targets of Y515 plays in the behavioural effects of VNS.

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Introduction

Major depressive disorder (MDD) is the single most frequent psychiatric disorder in the USA, with a lifetime prevalence of 16% in the adult population (Kessler et al., 2003). Many patients have a recurrence within 2–5 yr (Little, 2009). About 15% of these patients do not exhibit much clinical improvement, if any at all, with multiple treatments and are classified as having treatment resistant depression (TRD) (Berlim and Turecki, 2007). Some non-pharmacological interventions are currently being tested to treat TRD patients, e.g. electroconvulsive therapy (ECT), deep brain stimulation (DBS), repetitive transcranial magnetic stimulation (rTMS) (Shelton et al., 2010; Rosa and Lisanby, 2012) and vagal nerve stimulation (VNS), has been approved for TRD by the Food and Drug Administration (FDA) since 2005. Although an initial randomized controlled short-term study did not demonstrate efficacy of VNS in comparison with sham stimulation (Rush et al., 2005a), more recent long-term studies have found significant improvement in response and remission rates when VNS was given to TRD patients in addition to their treatment when compared with rates found in similar patients receiving just treatment as usual (George et al., 2005; Dunner et al., 2006; Schlaepfer et al., 2008). Furthermore, response to VNS not only increases with time but also is sustained over at least 2 yr (Bajbouj et al., 2010).

Because of this, there have been an increasing number of studies recently examining both behavioural and cellular effects in the brain after administration of VNS to rats, particularly in awake, freely moving animals. Activation of both noradrenergic and serotonergic neurons was observed in rats given VNS (Dorr and Debonnel, 2006; Furmaga et al., 2012a; Manta et al., 2013) with locus coeruleus activation occurring sooner than that in the dorsal raphe nucleus (Dorr and Debonnel, 2006). In relation to behavioural responses, VNS also caused reduced immobility in the forced swim test (FST), i.e. it had antidepressant-like activity and reduced the latency to feed in the novelty suppressed feeding test (NSFT), a measure of anxiolytic-like activity (Furmaga et al., 2011). These behavioural effects of VNS were not seen in rats with lesions of serotonergic or noradrenergic neurons (Furmaga et al., 2011).
In addition to effects on monoamines, it is now well established that antidepressants also affect the activity of neurotrophins such as brain-derived neurotrophic factor (BDNF) and can increase neurogenesis (Nibuya et al., 1995; Duman and Monteggia, 2006; Hodes et al., 2010). In light of this, it is worth noting that VNS can do this also (Follesa et al., 2007; Revesz et al., 2008; Biggio et al., 2009). We have been focusing on effects of VNS on the neurotrophin receptor, TrkB, in the brain. Both pre-clinical, imaging and post-mortem studies have indicated that either BDNF or TrkB may be involved in the pathogenesis of depression or as targets for antidepressant drugs (Saarelainen et al., 2003; Chen et al., 2005; Dwivedi et al., 2006). Both acute and chronic antidepressant treatments increase phosphorylation of TrkB at tyrosines 705 and 816 in brain of mice and rats (Rantamaki et al., 2007; Furmaga et al., 2012b). Interestingly, we also found that acute or chronic VNS treatment phosphorylates tyrosine 515 on TrkB (Furmaga et al., 2012b), which is a site distinct from those phosphorylated by standard antidepressant drugs. Because of the unique effects of VNS on phosphorylation of Y515 on TrkB, in the present study we characterized further activation of downstream pathways linked to specific key tyrosine residues on TrkB (namely Y816 and Y515) by either acute or chronic administration of VNS and conventional antidepressants. It was found that the pathway linked to Y515 was activated by VNS but not by standard antidepressants.

Method

Experiments were carried out using adult male Sprague-Dawley rats, 300–350 g (Harlan). Rats were group-housed and maintained in a temperature-controlled environment on a 14:10 h light–dark cycle and had access to food and water ad libitum. Experimental protocols were approved by the IACUC in accordance with the guidelines of the Public Health Service, American Physiological Society and the Society for Neuroscience.

Implantation of vagal nerve stimulators

Electrodes were implanted on the left vagus nerve under aseptic conditions. The surgical procedure was similar to that described by Furmaga et al. (2012b). 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 direct action potential propagation preferentially toward the central nervous system by creating an anodal block at the distal lead. The electrodes were connected to a stimulator pack (Cyberronics Inc., USA) 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 hand-held computer. Control rats received a dummy simulator pack that was the same size and weight (48 mm × 33 mm × 7.1 mm; 16 g). The rat was injected with penicillin immediately after surgery. Beginning 7 d after surgery, the stimulator was turned on for 0.5, 1 or 2 h (acute) or 14 d (chronic). The stimulation paradigm consisted of one burst of 20 Hz, 250 μs pulse width, 250 μA current for 30 s every 5 min. These parameters are the initial parameters used in clinical studies (Rush et al., 2005a, b), although parameters may change if patients do not respond. For both acute and the chronic studies, VNS stimulation occurred until just before the rats were sacrificed (see details in Western Blot analysis).

Implantation of osmotic mini-pumps

One day prior to surgery, osmotic mini-pumps delivering 5 μl/h (Model 2ML2, DURECT Corporation, USA) were filled with drug or vehicle, filtered through 0.9 μM nitrocellulose filters (Millipore, USA) using a sterile technique in an air-filtered hood. Drug solution concentrations were determined based on the mean rat weight over the 14 d of treatment. The doses used, 7.5 mg/kg/day of sertraline or 10 mg/kg/day desipramine, have been shown previously to produce serum concentrations in the therapeutic range (Benmansour et al., 1999). The vehicle solution consisted of 10% EtOH/0.9% NaCl for sertraline and distilled water for desipramine. Mini-pumps were stored in sealed containers filled with sterile saline overnight at room temperature to prime the pumps until the time of surgery. At the time of surgery, rats were anaesthetized with an intramuscular injection of a cocktail of 75 mg/kg ketamine and 0.5 mg/kg medetomidine. A drug-filled mini-pump was implanted intraperitoneally via a midline incision made on the lower abdomen. After the pump was implanted, the muscle and skin were sutured. The rat was injected with penicillin immediately after surgery. Rats were sacrificed while the mini-pumps were still dispensing drug.

Western blot analysis

Rats were sacrificed by rapid decapitation. For all groups, rats were anaesthetized with isoflurane prior to decapitation. For rats treated with VNS or sham stimulation, rats received an injection of 0.5 ml of 3 M KCl directly into the heart while still anaesthetized to allow the stimulating electrodes to be removed prior to decapitation. The electrodes were removed within 1 min of the injection of KCl. This allowed removal of the stimulators and leads immediately prior to decapitation. The brain was quickly removed and the entire hippocampus (bilateral) was dissected out on an ice-cooled dish. The dissected hippocampi were stored at −80 °C until processed for Western blot analysis. Samples were homogenized in a lysis buffer (50 mM Tris, 1 mM EDTA, 0.35% Na Deoxycholate, 150 mM NaCl, 1% Igepal, 0.1% SDS,
supplemented with protease and phosphatase inhibitor cocktail), incubated on ice for 30 min and then centrifuged (16,000 g, 20 min). Protein levels of the collected supernatant total lysate were measured using the Bradford assay (Biorad, USA). Proteins were separated in a 7.5% SDS-PAGE gel and blotted overnight (30 V) or for 1 h (100 V) onto a nitrocellulose membrane. Membranes were incubated at 4 °C overnight with the following primary antibodies: anti-pY515 (1:1000 in 2.5% BSA in TBST, Abcam, USA), anti-pY705 TrkB (1:1000 in 2.5% BSA in TBST, Abcam, USA), anti-pY816 TrkB (1:4000 in 2.5% BSA in TBST, Abcam, USA), and anti-TrkB (full length, 1:10000 in 2.5% BSA in TBST, Abcam, USA), anti-pY816 TrkB (1:4000 in 2.5% BSA in TBST, Abcam, USA), and anti-pp70 S6 kinase T389 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti- pp70 S6 kinase T389 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-pCREB S133 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-CREB (1:2000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-ERK (1:250 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-PLCγ1 (1:1000), anti-PLC γ1 Y783 (1:250 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-pERK T202/Y204 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-ERK (1:2000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-AKT (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-pAKT T308 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-CREB (1:2000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti-pCREB S133 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA), anti- pp70 S6 kinase T389 (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA) and anti-p70 S6 kinase (1:1000 in 5% BSA in TBST, Cell Signaling Technology, Inc, USA).

Equal loading was confirmed using anti-β actin (1:100,000 in 1% BSA in TBST, Sigma). Membranes were washed with TBS/0.1% Tween (TBST) and incubated with horseradish peroxidase conjugated secondary antibody (1:20000 in 1% BSA in TBST, Sigma, USA). Secondary antibodies tagged to peroxidase were used to visualize immunoreactive bands using enhanced chemiluminescence kits (Pierce, USA) followed by an exposure to x-ray film for detection. After obtaining the immunoreactive bands for the phosphorylated proteins, the respective antibodies were stripped from the nitrocellulose membrane using a commercially available stripping buffer (Thermo Scientific, USA), and then washed, blocked again and re-probed with antibodies for total protein or actin. Thus, phosphorylated protein, total protein and actin were obtained from the same Western blot for each phosphorylated target studied. Densitometry analyses of immunoreactive bands were performed using the NIH Image J software (USA). Results were calculated as the ratio of arbitrary densitometric units of phosphorylated target protein at specific tyrosine, threonine or serine residues to total non-phosphorylated target protein, and are shown graphically as the per cent, accordingly, of the sham VNS or vehicle values against total non-phosphorylated target. Total non-phosphorylated target protein arbitrary densitometric units were analysed separately against β actin. In no case did total protein levels change with the treatments, as revealed by analysis of total protein vs. actin. Thus, presenting the results as phosphoprotein divided by either total protein or actin generated the same outcomes. Consequently, we chose to present the results as phosphorylated protein divided by total protein.

**Statistical analysis**

Data for the phosphorylation of TrkB or PLCγ1 were analysed using multivariate analysis of variance (MANOVA) or ANOVA respectively, followed by Student’s Newman–Keuls post-hoc tests. For other results, data were analysed by the Mann–Whitney U test (if variances significantly different) or Student’s t-test. MANOVA was used as the phosphorylation sites for TrkB reside on the same protein and, therefore, are not independent measures. VNS data were analysed separately from drug data. p<0.05 was considered significant. All data are presented as the mean±S.E.M. percentage of control values. Number of animals per experiment varies from 6–12 per group.

**Results**

**Acute studies**

Previous work from our group (Furmaga et al., 2012b) demonstrated that acute (2 h) and chronic (14 d) administration of VNS caused phosphorylation of TrkB in hippocampus at three tyrosine sites (515, 705 and 816), whereas antidepressant drug treatments for the same times only caused phosphorylation of TrkB at the 705 and 816 sites. To address whether or not antidepressant drugs might be producing a transient change in phosphorylation at the 515 site, we carried out an analysis of TrkB phosphorylation after VNS and antidepressant treatments at earlier time-points. In the first experiment, the effect of VNS on TrkB phosphorylation was compared with that produced by acute administration of a selective norepinephrine reuptake inhibitor (NRI), desipramine (10 mg/kg, i.p.) at 30, 60 and 120 min post-treatment (Fig. 1a). MANOVA were carried out separately either for VNS treatment or for drug treatment. Desipramine produced a significant main effect of treatment [F(3, 28) =76.0, p<0.001] and time [F(6, 56)=15.7, p<0.001]. There was also an interaction between treatment and time [F(6, 56)=15.7, p<0.001]. Post-hoc analysis revealed that acute administration of DMI caused a significant increase in phosphorylation of tyrosines 705 and 816 at 60 and 120 min (Fig. 1a), but at no time point was an increase in phosphorylation of tyrosine 515 measured. Thus, even at time points earlier than 2 h, DMI caused no increase in phosphorylation at the Y515 site.

The effects of acute administration of VNS are shown in Fig. 1b. VNS produced a significant main effect of treatment [F(3, 28)=24.6, p<0.001] and time [F(6, 56)=15.4, p<0.001]. There was also an interaction between treatment and time [F(6, 56)=15.4, p<0.001]. Post-hoc analysis revealed that acute administration of VNS caused a significant increase in phosphorylation of
tyrosines 705, 816 and 515 at 120 min (Fig. 1b). However, at 30 or 60 min, there was no increase in phosphorylation at any site. Total TrkB protein levels were not altered after either acute antidepressant or VNS treatment (Fig. 1b), i.e. the ratio of total TrkB/β-actin for the sham group was not significantly different from that in the VNS group.

Because both VNS and DMI caused phosphorylation on the 816 site, and given that this site is associated with PLCγ1 activation (Blum and Konnerth, 2005; Rantamaki et al., 2007), Western blot analyses were carried out at various time points up to 2 h and phosphorylation of PLCγ1 at Y783 was measured. ANOVAs were carried out separately for VNS and DMI. Both VNS and DMI produced significant main effects of treatment [F(1, 30)=9.08, p<0.01 and F(1, 30)=7.73, p<0.05, respectively, for VNS and DMI] and time [F(2, 30)=13.31,
p<0.001 and $F(2, 30)=4.23$, $p<0.05$, respectively, for VNS and DMI]. There was also an interaction between treatment and time [$F(2, 30)=13.31$, $p<0.001$ and $F(2, 30)=4.23$, $p<0.05$, respectively, for VNS and DMI]. Post-hoc analyses revealed increased phosphorylation of PLC$_\gamma_1$ at 2 h, but not earlier, for both VNS and DMI (Fig. 2).

**Chronic studies**

We reported previously that chronic treatment with VNS, the SSRI sertraline, and DMI had identical effects on phosphorylation of TrkB at the three sites as found with acute administration, i.e. no effect of the drugs on Y515 but increased phosphorylation due to VNS (Furmaga et al., 2012b). Akt and ERK (Blum and Konnerth, 2005) are linked to Y515, whereas PLC$_\gamma_1$ is linked to Y816 (Blum and Konnerth, 2005). VNS, but not DMI or sertraline treatment, increased phosphorylation of AKT and ERK (Figs. 3 and 4, respectively). In contrast to what was found with acute administration, phosphorylation of PLC$_\gamma_1$ was not maintained with chronic administration of either VNS or the antidepressant drugs (Fig. 5). Total Akt, ERK and PLC$_\gamma_1$ protein levels were not altered after either chronic antidepressant or VNS treatment when compared with that measured in the control group (data not shown).

Phosphorylation of the transcription factor CREB is produced by chronic administration of antidepressants (Blendy, 2006). Many different pathways, including TrkB, can lead to increased phosphorylation of CREB (Blum and Konnerth, 2005). Consequently, Western blot analysis was carried out to measure the phosphorylation status of CREB after administration of VNS and the antidepressants. All treatments caused phosphorylation of down-stream signaling molecules (Furmaga et al., 2012b).
CREB after chronic administration (Fig. 6), but not after acute treatment at 30, 60 or 120 min (data not shown). As indicated in the method section, total CREB levels were not changed by any of the treatments at any time.

To investigate further the uniqueness of VNS in activating effectors primarily linked to the Y515 site on TrkB, we measured activation of the mTor pathway. AKT is the primary activator of the mTorC1 complex.
As a read out for the mTor pathway, p70S6kinase was chosen as it is known to be activated by mTorC1 (Pullen and Thomas, 1997; Franke, 2008). Chronic administration of VNS, but not chronic administration of the antidepressant drugs, increased phosphorylation of p70S6kinase at T783 (Fig. 7).

Discussion

The key finding in this study is that chronic administration of VNS not only causes phosphorylation of Y515 on TrkB but also phosphorylation of signaling molecules down-stream of this site. By contrast, chronic administration of the antidepressant drugs, increased phosphorylation of p70S6kinase at T783 (Fig. 7).

Fig. 5. Summary analysis of Western blot of hippocampus PLCγ1 (≈160 kDa) phosphorylation at Y783 after chronic 14 d VNS, sertraline (7.5 mg/kg/d i.p.) or DMI (10 mg/kg/d i.p.). The number of rats in each group is shown in the bars.

Fig. 6. Western blot analysis of hippocampus CREB (≈50 kDa) phosphorylation at S133 after chronic 14 d VNS, sertraline (7.5 mg/kg/d i.p.) or DMI (10 mg/kg/d i.p.). *p<0.01, Student’s t-test. The number of rats in each group is shown in the bars.

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Discussion

The key finding in this study is that chronic administration of VNS not only causes phosphorylation of Y515 on TrkB but also phosphorylation of signaling molecules down-stream of this site. By contrast, chronic administration of the traditional antidepressants, desipramine or sertraline, neither causes phosphorylation of Y515 on TrkB or that of down-stream signaling molecules linked to it. Even at time-points earlier than those examined by us previously (Furmaga et al., 2012b), DMI caused no phosphorylation of Y515. This latter result is in agreement with findings previously reported (Saarelainen et al., 2003; Rantamaki et al., 2007) in mouse brain, namely antidepressant drugs failed to cause phosphorylation of TrkB at the Y515 site. Interestingly, although both acute VNS and DMI produced phosphorylation of PLCγ1, which is linked to the site (Y816) on TrkB that is phosphorylated by both treatments, the increase in phosphorylation was not sustained after chronic administration of either treatment. Chronic administration of VNS did produce phosphorylation of CREB, an effect reported previously to occur following some antidepressant treatments (Nibuya et al., 1995; Duman, 1998; Saarelainen et al., 2003; Blendy, 2006; Rantamaki et al., 2007; Qi et al., 2008) and confirmed in this study with DMI.
Given the uniqueness of VNS to modulate targets linked to the Y515 residue on TrkB and its ability to treat resistant depression, we also studied molecular targets down-stream of the Y515 site on TrkB including Akt, ERK and p70S6 kinase. Phosphorylation of p70S6 kinase is mediated by the mTor pathway and reflects its activation (Zhou and Huang, 2010). P70S6 kinase is a particularly interesting target to examine in view of its activation by the fast-acting antidepressant ketamine (Li et al., 2010), which is also in used to treat resistant depression (Murrough, 2012; Murrough et al., 2013). Only VNS, but not DMI or sertraline, was found to promote p70S6 kinase phosphorylation in rat hippocampus.

Treatment resistant depression (TRD) is a major health problem in that about 15% of patients with major depression do not respond to treatment with conventional antidepressants (Kessler et al., 2003). That represents 3% of the United States adult population (Holtzheimer and Mayberg, 2012). Several treatment strategies have been examined for effectiveness in TRD including vagal nerve stimulation (VNS) (Shelton et al., 2010; Holtzheimer and Mayberg, 2012), which received FDA approval for TRD in 2005 following its approval 8 yr earlier for treatment resistant epilepsy (Nemeroff et al., 2006). Although efficacy of VNS was not found in an early 12-wk randomized control trial of TRD (Rush et al., 2005a), more recent longer-term studies have found efficacy (George et al., 2005; Rush et al., 2005b; Sackeim et al., 2007; Schlaepfer et al., 2008; Bajbouj et al., 2010; Cristancho et al., 2011) especially in comparison with TRD patients only receiving treatment as usual (Dunner et al., 2006; George et al., 2008; Schlaepfer et al., 2008). Patients who received VNS in addition to the conventional treatment alone showed response (27–53%) and remission rates (15–33%) that are considerably greater than those in TRD patients receiving just treatment as usual – 12% (response) and 4% (remission) (Dunner et al., 2006). Importantly, response to VNS not only increases with time, but also is sustained over at least 2 yr (Bajbouj et al., 2010; Rosa and Lisanby, 2012).

Another promising treatment for TRD that is under investigation both clinically and pre-clinically is the glutamate N-methyl-d-aspartate (NMDA) receptor antagonist, ketamine. Previous studies have shown that a single sub-anaesthetic dose of ketamine exerts fast-acting antidepressant effects in patients and in animal models with depression (Murrough, 2012; Murrough et al., 2013). The activity of the mammalian target of rapamycin (mTOR), a ubiquitously expressed serine/threonine kinase, is central to the regulation of translation initiation and, consequently, protein synthesis required for long-term potentiation and new synaptic connections. The activation of the mTOR signaling pathway is required for the rapid antidepressant actions of ketamine (Duman and Li, 2012). In view of these effects produced by ketamine and VNS in the clinic, the increased phosphorylation of the primarily mTor effector, p70S6kinase (Zhou and Huang, 2010) in the hippocampus found after chronic VNS treatment brings new perspectives as far as new targets for TRD treatments are concerned.
Both VNS and antidepressants increased phosphorylation at Y705 located at the kinase catalytic domain of TrkB and also at Y816 (Saarelainen et al., 2003; Rantamaki et al., 2007; Furmaga et al., 2012b). The primary consequence of tyrosine 816 phosphorylation is the physical interaction of this phosphoryresidue on TrkB with PLCγ1 (Blum and Konnerth, 2005), so as to produce phosphorylation of PLCγ1, for instance at its Y783 site (He et al., 2010). We studied such phosphorylation after either acute or chronic treatment with VNS or DMI by Western blot analysis using a commercial available antibody that recognizes the phosphorylated Y783 residue on PLCγ1. Increased phosphorylation of the Y783 residue on PLCγ1 was detected followed acute administration of VNS or DMI, whereas 14 d of treatment with VNS, DMI or sertraline failed to increase its phosphorylation. (Rantamaki et al., 2007) reported acute treatment of mice with fluoxetine did not increase the association of TrkB with PLCγ1, using a co-immunoprecipitation procedure. However, after chronic fluoxetine treatment for 21 d the same group found a significantly increased interaction between TrkB and PLCγ1 (Rantamaki et al., 2007). These results are different from ours. This may result from; a species (mouse vs. rat) difference, difference in route of drug administration (injections vs. mini-pumps) or differences in the methods used to analyse PLCγ1 activation, namely, co-immunoprecipitation vs. Western blot analysis of a specific phosphoresidue. Of interest, in our study the acute effects of DMI and VNS on PLCγ1 at Y783 did not persist over time despite the fact that all chronic treatments caused Y816 phosphorylation on TrkB.

Another molecular target for antidepressants is the transcription factor CREB. Different approaches have been used to measure the effect of antidepressant treatments on CREB in different brain areas, including measurement of its mRNA, immunohistochemistry, CRE binding, and protein analysis by Western blots for both CREB and phosphorylated CREB (at serine 133). Neither acute nor short-term (e.g. 3 d) treatment increased CREB or phospho-CREB (Nibuya et al., 1996; Thome et al., 2000; Tiraboschi et al., 2004). Consistent with such results, we did not see any increase in CREB or phospho-CREB 2 h after acute administration of VNS or DMI. Persistent antidepressant treatment, though, for 2–3 wk, does in general, increase either CREB and/or phospho-CREB, although the effects can vary in different brain regions (particularly prefrontal cortex vs. hippocampus) and with the antidepressant drug used (Nibuya et al., 1996; Thome et al., 2000; Tiraboschi et al., 2004; Laifenfeld et al., 2005; Qi et al., 2008; Tardito et al., 2009). Most studies in which it was examined reported increases in phospho-CREB (although in a brain region- and drug-specific manner) even if no changes in CREB were found (Qi et al., 2008; Tardito et al., 2009). Our data are consistent with these latter studies in that we found significant increases in the hippocampus in phospho-CREB after 14 d of drug or VNS treatment although no changes in total CREB were observed.

It is of some interest that effects of antidepressants on activation of TrkB and its down-stream signaling molecules may be brain region specific. This is because the functional roles of BDNF, at least with respect to mood regulation, can be opposite in different areas of brain (see Christoffel et al., 2011). In the hippocampus, for example, reduction in expression of BDNF induces depressive-like behaviour, whereas its expression and function is increased by antidepressant treatments (Nibuya et al., 1995; Shirayama et al., 2002; Saarelainen et al., 2003; Altar et al., 2004; Gronli et al., 2006; Rantamaki et al., 2007; Taliiaz, et al., 2010). By contrast, ablation of BDNF from the ventral tegmental area (VTA) of mice produces an antidepressant-like effect (Berton et al., 2006), and a similar result was produced when a dominant negative mutant of TrkB was over-expressed in the nucleus accumbens (NAC) (Eisch et al., 2003). Also, the antidepressant-like effect of ECT is not blocked by lentiviral injection-induced knockdown of BDNF in the hippocampus. However, ECT reduced BDNF levels in the VTA and knocking down BDNF here caused an antidepressant-like effect (Taliiaz et al., 2013). Data such as these need to be kept in mind as the present experiments were carried out exclusively using hippocampal tissue.

More recent studies have also suggested that CREB is implicated in the cellular effects of another adjunctive therapy for TRD, namely repetitive transcranial magnetic stimulation (rTMS) (Hellmann et al., 2012). Moreover, the positive effects of rTMS upon CREB phosphorylation were potentiated by ketamine pre-treatment (Hellmann et al., 2012). Thus, chronic administration of stimulation therapies that have efficacy in TRD produce increased phosphorylation of CREB.

Phosphorylation of Akt or ERK was not increased after either acute or chronic traditional antidepressant treatment. Both acute and chronic VNS treatment, on the other hand, caused phosphorylation of Akt or ERK. This is consistent with the activation of Y515 phosphorylation by VNS treatment and the lack of Y515 phosphorylation by antidepressant treatments. Previous data on antidepressant drugs effects on ERK phosphorylation are in agreement to those found in the present study (Rantamaki et al., 2007; Di Benedetto et al., 2012; First et al., 2013). By contrast, both Qi et al., (2008) and Tardito et al., (2009) reported that chronic administration of fluoxetine to rats did increase phospho-ERK2 or phosph-ERK1/2 but not the non-phosphorylated proteins in the hippocampus. The reason for these discrepant results is not clear.

It is of some interest that neither DMI nor sertraline increased phosphorylation of ERK or Akt or p70S6 kinase. Although this is consistent with these drugs not phosphorylating Y515 on TrkB, there are other cellular
mechanisms by which these signaling molecules can be activated and some of these involve biogenic amines, which are known to be affected by antidepressants. However, the best evidence for regulation of AKT by biogenic amines is related to dopamine (Beaulieu et al., 2009; Beaulieu, 2012) and even then the dopamine 2 receptor (D2 receptor) is inhibitory to AKT. Also, there is little evidence that noradrenaline (NE) or serotonin (5-HT) reuptake inhibitor antidepressants, such as desipramine or sertraline, respectively, inhibit the uptake of dopamine so as to alter dopaminergic signaling (Bolden-Watson and Richelson, 1993). Further, there is little evidence that activation of serotonin receptors, as would occur following chronic administration of sertraline, causes activation of AKT or ERK (Beaulieu et al., 2009; Beaulieu, 2012). Activation of 5-HT1A and 5-HT2A receptors may regulate GSK3β activity (Li et al., 2004, 2007), but GSK3β is downstream from AKT. We have not been able to find data linking activation of noradrenergic receptors, as would occur with chronic administration of DMI, to regulation of AKT in vivo. There is a report where acute administration of NE to embryonic primary hippocampal neurons in culture increased phosphorylation of PI-3kinase, ERK1/2 and CREB (Chen et al., 2007). However, it is not clear if this occurs in vivo in the adult.

Traditional antidepressants have been known to work by targeting noradrenergic and/or serotonergic neurons (Haenisch and Bonisch, 2011). VNS also activates these neurons (Manta et al., 2009, 2013; Furmaga et al., 2012a). These neurons seem to be important to the behavioural effects of VNS (Furmaga et al., 2011). Although traditional antidepressants activate TrkB, the present study shows that VNS not only activates TrkB at sites phosphorylated by traditional antidepressants but also an additional site. Furthermore, signaling molecules specific to this site are affected by VNS and not traditional antidepressants. Thus, VNS has additional effects on TrkB, namely it induces phosphorylation of the juxtamembrane site Y515, and down-stream signaling linked to that site. The involvement of this additional pathway activated by VNS in its behavioural effects remains to be elucidated but it does highlight differences between VNS and traditional antidepressants while showing a similarity in relation to the effects of VNS and ketamine.

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Statement of Interest

Dr Carreno has no financial disclosures to report. Dr Frazer has served on advisory boards for Lundbeck and for Takeda Pharmaceuticals International, Inc and Eli Lilly and Co. Previously, Dr Frazer had received financial compensation as a consultant for Cyberonics Inc. and had also obtained grant support from them for a preclinical study.

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