Published in final edited form as:
Int J Neuropsychopharmacol. 2009 October ; 12(9): 1209–1221. doi:10.1017/S1461145709000200.

Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus

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Abstract

Vagus nerve stimulation (VNS) is used to treat pharmacotherapy-resistant epilepsy and depression. The mechanisms underlying the therapeutic efficacy of VNS remain unclear, however. We examined the effects of VNS on hippocampal neuronal plasticity and behavior in rats. Cell proliferation in the hippocampus of rats subjected to acute (3 h) or chronic (1 month) VNS was examined by injection of bromodeoxyuridine (BrdU) and immunohistochemistry. Expression of doublecortin (DCX) and brain-derived neurotrophic factor (BDNF) was evaluated by immunofluorescence staining. The dendritic morphology of DCX^+ neurons was measured by Sholl analysis. Our results show that acute VNS induced an increase in the number of $BrdU^+$ cells in the dentate gyrus that was apparent 24 h and 3 weeks after treatment. It also induced long-lasting increases in the amount of DCX immunoreactivity and the number of DCX^+ neurons. Neither the number of $BrdU^+$ cells nor the amount of DCX immunoreactivity was increased 3 weeks after the cessation of chronic VNS. Chronic VNS induced long-lasting increases in the amount of BDNF immunoreactivity and the number of $BDNF^+$ cells as well as in the dendritic complexity of DCX^+ neurons in the hippocampus. In contrast to chronic imipramine treatment, chronic VNS had no effect on the behavior of rats in the forced swim or elevated plus-maze tests. Both chronic and acute VNS induced persistent changes in hippocampal neurons that may play a key role in the therapeutic efficacy of VNS. However, these changes were not associated with evident behavioral alterations characteristic of an antidepressant or anxiolytic action.

Keywords

Neurotrophines; Neurogenesis; Gene expression; Antidepressant drugs; Treatment-resistant depression

Introduction

A vagus nerve stimulation (VNS) device consists of an implantable generator connected to electrodes that deliver chronic low-frequency electrical signals to the left cervical vagus nerve.

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Statement of Interest
None

Intermittent VNS with such a device has become a well-established, safe, and effective adjunct to medical therapyfor refractory epilepsy (Ben-Menachem, 2002; Schachter, 2006). Observations of mood elevation during VNS therapy for pharmacoresistant epilepsy (Ben-Menachem, 2002; Elger et al., 2000; Harden, 2002; Harden et al., 2000) suggested that such treatment might also show efficacy for refractory major depression. The mood improvement was found to be sustained and independent of antiseizure action (Elger et al., 2000; Harden et al., 2000). Surrogate markers of mood alteration, such as psychosocial function, attention, memory, temperament, and the ability to cooperate, were also shown to be improved by VNS (Ben-Menachem et al., 1994; Clark et al., 1999; Milby et al., 2008; Schlaepfer et al., 2008). On the basis of such observations, the VNS device was recentlyalso approved for the treatment of resistant depression (George and Sackeim, 2008; George et al., 2000; Goodnick et al., 2001), and some clinical studies show antidepressant efficacy in patients with treatment-resistant depression (George et al., 2005; Nahas et al., 2005; Rush et al., 2005; Schlaepfer et al., 2008). Nevertheless, up-to-date there is still debate about the effectiveness of VNS therapy with controversy ranging from conflicts of interest to questionable reporting (Carlat, 2006; Lurie and Stine, 2006). Moreover, as in epilepsy, the mechanisms underlying the putative efficacy of VNS therapy for drug-resistant depression have remained unclear.

Neuroimaging and other neurobiological studies have suggested that activation of the nucleus tractus solitarius plays a keyrole in VNS therapy. This structure sends projections to brain areas implicated in modulation of affective state (Henry, 2002), including secondary projections to limbic and cortical structures such as regions of the brainstem that contain serotonergic (raphe nucleus) and noradrenergic (locus ceruleus) perikarya that project to the forebrain. Given the important role that norepinephrine and serotonin play in modulation of emotional and affective behavior, the activation of nucleus tractus solitarius projections to the locus ceruleus and raphe nucleus might be relevant to the therapeutic efficacy of VNS. The firing rates of neurons in the rat dorsal raphe nucleus and locus ceruleus have been shown to be increased after long-term treatment with VNS (Dorr and Debonnel, 2006; Krahl et al., 1998), whereas depletion of norepinephrine in the locus ceruleus abolished the seizure-suppressive effect of VNS (Krahl et al., 1998). Furthermore, the concentration of serotonin metabolites was found to be increased in the cerebrospinal fluid of patients treated with VNS (Ben-Menachem et al., 1995), and we previously showed that acute VNS, like antidepressant drugs (Dazzi et al., 2002a; Dazzi et al., 2002b), increased the concentration of norepinephrine in the rat prefrontal cortex (Follesa et al., 2007). These various observations support the idea that VNS acts directly by stimulating brainstem structures and indirectly by regulating the activity of neurons in limbic and cortical regions involved in mood modulation. This conclusion is further supported both by functional magnetic resonance imaging studies of depressed patients showing VNS-induced bilateral increases in blood oxygenation level in various brain regions implicated in mood disorders and regulated by the vagus nerve (Bohning et al., 2001; Lomarev et al., 2002) as well as by positron emission tomography–based studies in humans (Conway et al., 2006; Henry et al., 2004; Pardo et al., 2008) and rat (Dedeurwaerdere et al., 2005b).

Despite their induction of a rapid increase in the extracellular levels of serotonin or norepinephrine in the brain, most antidepressant drugs exhibit clinical efficacyonly after treatment for at least 3 to 4 weeks (Wong and Licinio, 2001). This delay is thought to reflect late neurochemical and structural changes in neurons within limbic target areas. Chronic, but not acute, treatment with antidepressants thus induces plastic and trophic effects that are thought to be necessary for a reduction in the vulnerability to negative environmental stimuli (Duman, 2005; Duman et al., 1997; Duman and Monteggia, 2006). All these observations lead to the neurotrophic hypothesis of depression, according to which decreased levels of neurotrophic factors, most notably brain derived neurotrophic factor (BDNF), contribute to the hippocampal atrophy seen in depressed patients, and antidepressant treatments achieve their therapeutic effects through increased expression of neurotrophic factors in the hippocampus

(Duman and Monteggia, 2006) promoting proliferation and then maturation of doublecortin (DCX) positive neurons (Wang et al., 2008). Accordingly, hippocampal neurogenesis is a requirement for the behavioral effects of antidepressants (Santarelli et al., 2003).

In an attempt to provide insight into the mechanisms underlying the putative therapeutic efficacy of VNS in patients with drug-resistant epilepsy or depression, we have studied an animal model to identify the brain regions affected and the associated neurochemical changes induced by VNS. We previously showed that acute VNS increases the expression of growth factors in the rat cerebral cortex and hippocampus as well as the release of norepinephrine in the medial prefrontal cortex (Follesa et al., 2007). We have now examined the effects of chronic VNS on hippocampal cell proliferation as well as on the expression of DCX and BDNF in rat brain and whether such effects might be associated with behavioral changes similar to those observed after chronic treatment with antidepressant drugs (Porsolt et al., 2001; Porsolt et al., 1977).

Methods

Animals and surgical procedure

All animal procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were performed with adequate measures to minimize pain or discomfort. The experimental protocols were also approved by the Animal Ethics Committee of the University of Cagliari. Male Sprague-Dawley rats (body mass, 250 to 300 g) housed under standard laboratory conditions were anesthetized by intraperitoneal (i.p.) injection of Equithesin (Deacon and Rawlins, 1996) at a dose of 1 ml per 300 g of body mass. A VNS therapy stimulator (Cyberonics, Houston, TX) was implanted into rats as previously described (Follesa et al., 2007). In brief, an incision was made at the left side of the ventral neck, the left vagus nerve was identified, and the helical bipolar leads were carefully wrapped around the left vagus nerve (Follesa et al., 2007; Handforth and Krahl, 2001). The leads were tunneled toward a horizontal incision on the back and connected to the pulse generator (Cyberonics). A control group of animals was subjected to the same surgery with leads that were connected to a dummy pulse generator. Two days after surgery, the pulse generator was activated for acute (3 h) or chronic (1 month) treatment according to stimulation parameters similar to those used in humans (Dedeurwaerdere et al., 2004; Marrosu et al., 2003; Sackeim et al., 2001) and in rats (Dedeurwaerdere et al., 2005b; Follesa et al., 2007): 30 s ON, 5 min OFF; continuous cycle; pulse frequency, 30 Hz; pulse time, 500 μs; pulse amplitude, 1.5 mA.

Drug treatment

Groups of naïve rats were treated for 1 month with imipramine (10 mg/kg, i.p., twice daily) or saline as controls for the behavioral tests. Proliferating cells in the dentate gyrus of rats subjected to acute or chronic VNS were labeled by injection with bromodeoxyuridine (BrdU). Animals subjected to acute VNS for 3 h and matching sham-operated controls were injected with a single dose of BrdU (100 mg/kg, i.p.) 30 min before the end of VNS and were killed either 24 h or 3 weeks after the end of stimulation. Animals subjected to chronic VNS for 1 month and matching sham-operated controls were injected with BrdU (70 mg/kg, i.p.) four times at intervals of 4 h during the final 12 h of stimulation and were killed 3 weeks after the end of stimulation.

Behavioral tests

For all behavioral experiments rats were tested in a randomized order by an operator blind to the rat tested status.

The elevated plus-maze test was performed as described (Follesa et al., 2002) but with the use of a video tracking system (Noldus et al., 2001). The plus maze was constructed of black polyvinyl chloride and comprised two open and two closed arms (12 by 60 cm) connected by a central square (12 by 12 cm) that served as the start point. The apparatus was mounted 50 cm above the floor of a quiet and dimly lit room. Rats subjected to chronic VNS or injected with imipramine, as well as corresponding numbers of respective controls, were allowed to adapt to the experimental room for 1 h before the test. Each animal was placed at the start point of the maze facing an open arm. The number of entries into open and closed arms as well as the time spent in each type of arm were then monitored for 5 min. Rats were tested in a randomized order between 09.00 and 14.00 hours. Video images of each rat were analyzed with the EthoVision 3.1 video analysis system (Noldus Information Technology, Wageningen, the Netherlands).

The forced swim test was also performed as described previously (Porsolt et al., 2001; Porsolt et al., 1977). The apparatus consisted of a Plexiglas cylinder (50 cm in height, 30 cm in diameter) that was filled with water at 25°C to a height of 40 cm. Rats subjected to chronic VNS or injected with imipramine, as well as corresponding numbers of respective controls, were allowed to adapt to the experimental room for 1 h before the test. Each rat was also subjected to a trial run of the test for 15 min on the day before the actual test, which was performed for 5 min. Rat behavior was analyzed with the use of a video tracking system (Noldus et al., 2001), and video images were analyzed with the use of the EthoVision 3.1 video analysis system. The times of immobility, mobility (swimming), and high mobility (climbing) were measured.

Immunostaining

Animals were anesthetized with Equithesin (Deacon and Rawlins, 1996) as described above and then perfused transcardially with 4% paraformaldehyde. The brain was removed, exposed to 4% paraformaldehyde, and transferred to buffered solutions of sucrose [30% (w/v)], and serial cross-sections (16 μm) of the dorsal hippocampus (Paxinos and Watson, 1982) were prepared and stored at −80°C until processing for immunohistochemical or immunofluorescence analysis. On the day of processing, sections were thawed at room temperature and hydrated in phosphate-buffered saline (PBS). BrdU-positive cells were detected by immunohistochemistry (Eriksson et al., 1998; Ming and Song, 2005). Tissue sections were first subjected to DNA denaturation (Kuhn et al., 1996) and were then treated with 0.3% H_2O_2 and 0.3% goat serum in PBS for 5 min at room temperature to block endogenous peroxidase activity. They were incubated for 30 min at room temperature with PBS containing 0.3% Triton X-100 and 5.5% goat serum and then overnight at 4°C with mouse monoclonal antibodies to BrdU (Roche Diagnostic, Monza, Italy) at 2 μg/ml in PBS containing 2% bovine serum albumin, 0.3% Triton X-100, and 5.5% goat serum. They were then washed with PBS before incubation for 1 h at room temperature with biotinylated goat secondary antibodies (Vector Laboratories, Peterborough, UK). Immune complexes were detected with a Vectastain peroxidase system (Vector Laboratories) including the peroxidase substrate diaminobenzidine with NiCl. Sections were counterstained with mouse monoclonal antibodies to NeuN (Millipore, Billerica, MA) at a dilution of 1:1000, biotinylated goat secondary antibodies (Vector Laboratories), and a Vectastain peroxidase system as above but with NovaRed as the substrate. The sections were finallymounted with a nonaqueous mounting medium and examined with a with a Zeiss (Carl Zeiss, Munich, Germany) Axioplan2 microscope equipped with a video camera. The numbers of BrdU-positive cells in the granule cell layer and the subgranular zone (the region including two cell diameters below the granule cell layer) of the dentate gyrus were determined in 24 coronal sections (16 μm in thickness and separated by 96 μm) from each brain. Cells in the uppermost focal plane were ignored byfocusing through the thickness of the section (optical dissector principle) (Coggeshall and

Lekan, 1996) in order to avoid oversampling errors. Data are expressed as BrdU-positive cells per sample volume per section.

BDNF and DCX were detected by immunofluorescence analysis of tissue sections with rabbit polyclonal antibodies to BDNF (Santa Cruz Biotechnology, Santa Cruz, CA) and guinea pig polyclonal antibodies to DCX (Millipore) as well as with corresponding secondary antibodies labeled with Alexa Fluor 488 (Invitrogen, San Giuliano Milanese, Italy) or with Texas red (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.), both at a dilution of 1:1000. Sections stained for DCX were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or NeuN, as indicated. All sections were mounted with the use of Vectashield mounting medium (Vector Laboratories) and then examined with a Zeiss (Carl Zeiss) Axioplan2 fluorescence microscope equipped with a 10× or 20× objective and AxioVision 40 V 4.6.3.0 software (Carl Zeiss). Twelve sections (thickness of 16 μm and separated by 96 μm) of the dorsal hippocampus were examined per animal. For quantitation of DCX and BDNF expression in the dorsal dentate gyrus or CA3 region, respectively, the total fluorescence intensitycorresponding to each region was measured with the use of AxioVision software in a series of 24 digitized microscopic images (two per section, left and right) for each rat; the intensity values were then averaged for each group of animals and expressed as the mean densitometric gray-scale value. The mean number of DCX^+ or $BDNF^+$ cells per field was also determined.

Sholl analysis

To measure the extent of dendritic growth away from the soma and the branching of dendrites at various distances from the soma, we performed concentric analysis of Sholl (Sholl, 1953) with the use of ImageJ 1.40g software (NIH, Bethesda, MD). The analysis was performed on 24 images for each animal at 20× magnification obtained with a Zeiss (Carl Zeiss) Axioplan2 fluorescence microscope equipped with a video camera.

Statistical analysis

Data are presented as means ± SEM and were compared by one-way analysis of variance (ANOVA) and Scheffe's test with the use of Statistica software (StatSoft, Tulsa, OK), with the exception that Sholl analysis comparisons were performed by two-way ANOVA for repeated measures and Newman-Keuls test. A P value of <0.05 was considered statistically significant.

Results

Effects of acute VNS on cell proliferation and DCX immunoreactivity

To examine the effect of acute VNS for 3 h (Follesa et al., 2007) on the proliferation of cells in the dentate gyrus of the hippocampal formation, we injected rats with BrdU 30 min before the end of VNS and detected the labeled cells by immunohistochemical analysis either 24 h or 3 weeks after the end of stimulation. In animals killed 24 h after acute VNS, the number of $BrdU^+$ cells in the dorsal dentate gyrus was significantly increased (2200 ± 159; $P < 0.05$) compared with that apparent in sham-operated controls (1760 ± 74) (Figure 1A). These cells were located in the subgranular zone and appeared as clusters of dividing cells (Figure 1C). The number of $BrdU^+$ cells remained increased 3 weeks after the acute stimulation (2448 ± 129; $P < 0.01$) (Figure 1B), but the labeled cells were now located in the inner granule cell layer, rather than in the subgranular zone as observed 24 h after the treatment, and they were no longer grouped in clusters (Figure 1D).

The increase in the number of $BrdU^+$ cells apparent 3 weeks after acute VNS was accompanied by an increase in the total amount of DCX immunoreactivity in the dorsal dentate gyrus (Figure

2A). Quantitative analysis revealed that acute VNS significantly increased both the total amount of DCX immunoreactivity (+39%; $P < 0.05$) (Figure 2B) as well the number of DCX^+ neurons (+57%; $P < 0.01$) (Figure 2C) compared with those apparent in sham-operated control rats.

Effects of chronic VNS on cell proliferation, DCX immunoreactivity, and dendritic complexity

To examine the effect of chronic VNS for 1 month on the proliferation of cells in the dentate gyrus, we injected rats with BrdU on the final day of stimulation and detected the labeled cells by immunohistochemical analysis 3 weeks later. The number of $BrdU^+$ cells (1857 ± 93) in the dorsal dentate gyrus of animals subjected to chronic VNS did not differ significantly ($P = 0.3546$) from the number of cells (1956 ± 52) in those animals subjected to sham surgery (Figure 3A). Moreover, the amount of DCX immunoreactivityin the dorsal dentate gyrus did not differ significantly ($P = 0.6081$) between these two groups of rats (757 ± 146 and 646 ± 154 respectively) (Figure 3B).

To examine further the effects of VNS on DCX^+ neurons in the dorsal dentate gyrus, we evaluated the complexity of their dendrites 3 weeks after acute or chronic stimulation by Sholl analysis. Such analysis revealed that both acute and chronic VNS altered the dendritic morphology of DCX^+ neurons. In particular, acute VNS significantly ($P < 0.05$ and $P < 0.01$) increased the complexity of dendrites by increasing the number of intersections (from 1.7 to 3.0) at distances between 60 and 80 μm from soma (Figure 3C), whereas the number of intersections at distances between 100 and 170 μm from the soma was significantly greater ($P < 0.001$) in rats subjected to chronic VNS than in sham-operated controls (4.1 and 1.6 respectively) (Figure 3C and E–F).

Moreover, the length of dendrites that project into the molecular layer of the hippocampus was significantly greater (156 ± 9 μm; $P < 0.05$) in rats subjected to chronic VNS than in those subjected to sham surgery (120 ± 9 μm) (Figure 3D and E–F). The nucleus of most DCX^+ cells was also positive for NeuN (Figure 3E and F).

Effects of chronic VNS on hippocampal BDNF immunoreactivity

The changes in hippocampal cytoarchitecture induced by chronic VNS were accompanied by effects on the expression of BDNF. Immunostaining for BDNF in the CA3 region of the hippocampus was thus markedly increased 3 weeks after chronic VNS, with the immunoreactivity being widely distributed among cell bodies and fibers (Figure 4A). Quantitative analysis revealed that both the amount of BDNF immunoreactivity (Figure 4B) and the number of $BDNF^+$ cells per field (Figure 4C) were significantly greater (+104% and +40% respectively; $P < 0.001$) in rats subjected to chronic VNS than in sham-operated animals.

Effects of chronic VNS on behavior

To determine whether chronic VNS induces behavioral effects similar to those elicited by classical antidepressant drugs, we first compared performance in the forced swim test between rats subjected to chronic VNS and those subjected to chronic treatment with imipramine. Chronic VNS did not significantly affect the times of immobility or high mobility in this test (Figure 5A), whereas these parameters were significantly reduced (−58%; $P < 0.01$) and increased (+20%; $P < 0.05$), respectively, in rats treated for 1 month with imipramine (Figure 5B). We further evaluated whether chronic VNS might exert an anxiolytic effect with the use of the elevated plus-maze test. Chronic VNS did not affect the time spent in the open arms or at the start point (Figure 5C), whereas, as expected (Pinheiro et al., 2008; Teixeira et al., 2000), chronic treatment with imipramine significantly increased (about 5 folds; $P < 0.05$) the time spent in the open arms and tended to increase that at the start point (Figure 5D).

Discussion

Our results have shown that both acute and chronic VNS induced long-term changes in hippocampal neurons. These changes in the hippocampus included an increase in the number of proliferating cells, which was observed only in acute VNS treated rats, and an increase in the complexity of dendritic arborization of DCX^+ neurons, observed in both acute and chronic VNS treatment. Chronic VNS also induced an increase in the extent of BDNF expression. Even though some of these effects reminiscent of those induced by treatment with antidepressant drugs (Schmidt and Duman, 2007; Wang et al., 2008), the effects of chronic VNS in the hippocampus were not associated with behavioral alterations as assessed by the forced swim test or elevated plus-maze test.

Effects of acute VNS

We previously showed (Follesa et al., 2007) that acute VNS increased the expression in the rat hippocampus of genes for BDNF and basic fibroblast growth factor, both of which are important modulators of hippocampal plasticity and neurogenesis (Duman and Monteggia, 2006; Raballo et al., 2000; Santarelli et al., 2003; Schmidt and Duman, 2007; Zhao et al., 2007). Consistent with these findings, we have now demonstrated that acute VNS induced an increase in the number of $BrdU^+$ cells in the dentate gyrus, a brain region that has been shown to harbor neuronal stem cells in adult mammals (Ming and Song, 2005) including humans (Eriksson et al., 1998). The increase in the number of $BrdU^+$ cells observed in our experimental paradigm is in agreement with a very recent study (Revesz D, 2008) showing rapid effects of VNS on rat hippocampal progenitor proliferation and correlated well with our observation that acute VNS also increased both the total amount of DCX immunoreactivity and the number of DCX^+ neurons in the dentate gyrus. The expression of DCX is in fact considered an index of hippocampal neurogenesis (Couillard-Despres et al., 2005) and has been used as a marker to analyze both the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus (Rao and Shetty, 2004).

Thus, at variance with acute antidepressant drugs (Duman et al., 1997; Wang et al., 2008; Wong and Licinio, 2001), acute VNS affects cell proliferation and hippocampal plasticity. Similar to VNS (Revesz D, 2008), electroconvulsive therapy stimulates cell proliferation more rapidly than do antidepressant drugs (Warner-Schmidt and Duman, 2007). The observed effects of acute VNS remained apparent 3 weeks after treatment and might therefore play a role in reshaping cellular networks underlying inhibitory processes (Clark et al., 1999; Marrosu et al., 2003) or in triggering intracellular events that could increase sensitivityto therapeutic drugs. Thus, acute VNS might therefore accelerate events that usually occur over a period of weeks in response to antidepressants and thereby potentiate the effects of such drugs. Given that in the human studies VNS is associated with the usual pharmacological treatment, it will be important to test the short-term effects of antidepressant treatment in association with VNS on hippocampal plasticity and neurogenesis.

Effects of chronic VNS

In contrast to the stimulatory effects of acute VNS, chronic VNS did not alter the numbers of $BrdU^+$ or DCX^+ cells in the dentate gyrus of the rat hippocampal formation. However, Sholl analysis revealed that, whereas acute VNS increased the arborization of DCX^+ neurons at distances of 60 to 80 μm from the soma, chronic VNS did so at distances of 100 to 170 μm. These effects on dendritic complexity induced by both acute and chronic VNS were long lasting in that they were detected 3 weeks after the end of treatment, and theywere similar to those induced by chronic treatment with fluoxetine (Wang et al., 2008). Moreover, consistent with previous observations showing increased expression of BDNF in response to antidepressant treatment in animal models of depression (Nibuya et al., 1995), we found that chronic VNS

induced a long-lasting increase in the expression of this neurotrophic factor in the CA3 region of the hippocampus. This increased expression of BDNF may serve to promote and maintain new neuronal connections formed in response to chronic VNS (Lipsky and Marini, 2007). Accordingly, the granule cells of the hippocampal dentate gyrus synapse throughout the mossy fiber pathway with the CA3 subfield of the hippocampus (Paxinos, 1995) where we observed the increase in BDNF expression.

Our finding that an effect of chronic VNS on cell proliferation was not apparent 3 weeks after the end of treatment appears at variance with the effect of chronic treatment with antidepressants (Santarelli et al., 2003) and suggests that chronic VNS promote the survival and trophism of the new cells, generated in the early phases of stimulation (see acute effects), rather then increses cell proliferation indefinitely. Therefore, given that remains unknown whether a persistent increase in cell proliferation and neurogenesis could be potentially dangerous, the short-term effect of VNS on cell proliferation might prove to be an advantage of VNS over antidepressant drug treatment. Thus, although adult neurogenesis is thought to add an additional layer of plasticity to hippocampal circuitry (Bruel-Jungerman et al., 2007), future studies on the biological consequences of persistent neurogenesis should help to elucidate whether and how this process might contribute to disease pathophysiology (Parent, 2008).

Lack of behavioral effects of chronic VNS

Whereas a previous study (Krahl et al., 2004) showed that sub acute treatment with VNS reduced the immobilitytime in the forced swim test, an important indicator of antidepressant action, we found that chronic VNS, in contrast to chronic treatment with imipramine, did not induce significant behavioral changes related to antidepressant or anxiolytic action in the forced swim or elevated plus-maze tests. This apparent discrepancy might be due to the high sensitivityof the Wistar-Kyoto strain of rats used in this previous study to the depressogenic effect of the forced swim test (Pare, 1989). The parameters of stimulation used in our study also differed from those used in the previous study. In addition, the antidepressant effect of VNS in the previous study was observed after 4 days of stimulation for 30 min per day and with the stimulator off during the forced swim test, whereas, in our study, the rats were stimulated for 1 month and the stimulator was still active during the test. Nevertheless, as suggested by the dramatic rearrangements of the neuronal network in the hippocampus of our animals after chronic VNS, we cannot role out the possibility that this negative result could be interpreted, rather then a mere lack of antidepressant action, as a smarter performance of these animals that better remember, as opposed to sham operated rats, the experience of the pre-test administered twenty-four hours earlier. We are currently testing this hypothesis by using the water-maze test.

Conclusions

We have examined the effects of chronic VNS to determine whether they differ from those of acute VNS in experimental animals. We found that acute VNS induced an immediate increase in the number of $BrdU^+$ cells in the dentate gyrus that was still apparent 3 weeks after treatment. This effect was similar to that induced by electroshock (Warner-Schmidt and Duman, 2007) and was much faster than that induced by antidepressant drugs, which require at least 2 weeks to induce cell proliferation (Santarelli et al., 2003; Warner-Schmidt and Duman, 2007). In contrast, an effect of chronic VNS on cell proliferation was not apparent 3 weeks after the termination of stimulation. Positron emission tomography previously showed that VNS with the same parameters as those used in our study was associated with a decrease in glucose metabolism in the hippocampus of rats after acute treatment but not after chronic treatment (Dedeurwaerdere et al., 2005a). We found that chronic VNS did induce a robust increase in

the expression of BDNF in the hippocampus, which may play an important role in consolidating the changes in neuronal connections revealed by the increased complexity of the dendritic arborization of DCX^+ neurons induced by this treatment. This latter effect of chronic VNS was similar to that induced by chronic treatment with an antidepressant drug (Wang et al., 2008). VNS treatment in drug-resistant patients is delivered in association with pharmacological therapy, and both the immediate and long-term effects of VNS on neurons may facilitate the action of antidepressant or antiepileptic drugs. Chronic (1 year) VNS has previously been shown to up-regulate the expression of γ-aminobutyric acid type A ($GABA_A$) receptors in putatively epileptogenic areas of the cortex (Marrosu et al., 2003) as well as to enhance memory retention (Clark et al., 1999) in humans.

Thus, although further clinical and experimental studies are necessary to determine the mechanisms of action of VNS in the treatment of epilepsy or depression, our results suggest that the promotion of neurogenesis may play an important role. Whether such newly generated neurons contribute to existing or de novo networks that mediate antiepileptic or antidepressant effects also remains to be determined.

Acknowledgments

This study is supported in part by the Italian Ministry of Instruction, University, and Research (M.I.U.R.), Project Center of Excellence for the Neurobiology of Dependence, D.M. January 2001, Grant protocol no. CE00042735 and in part by HHS grant NS047977. Cyberonics, Inc., manufacturer of the VNS Therapy System, provided the authors with VNS devices adapted for use in animals. The authors did not receive compensation from the manufacturer for performing this research or preparing the paper.

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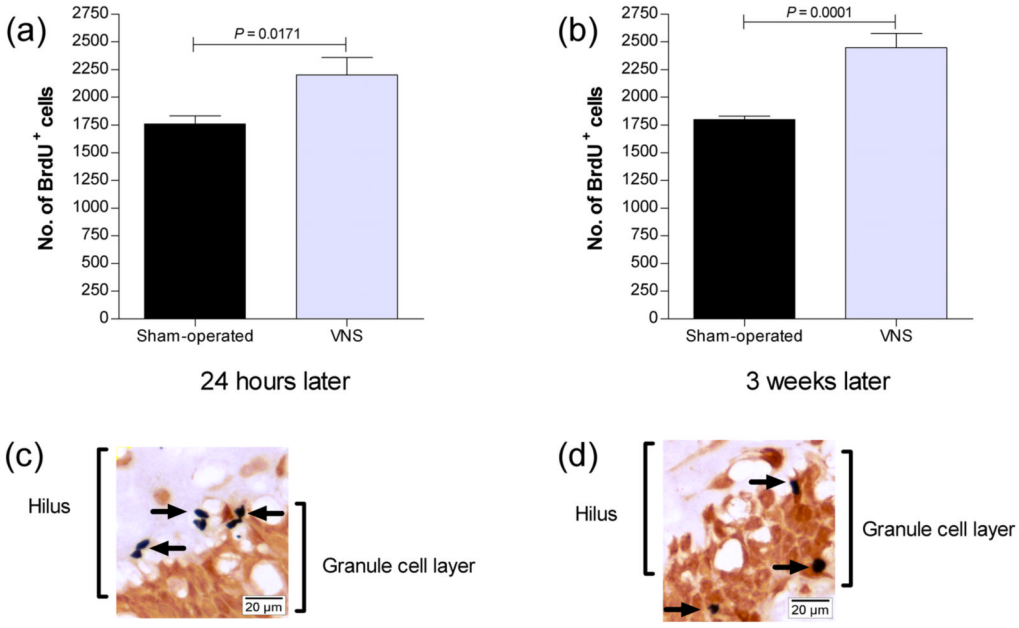

Figure 1.

Quantitation of newly generated cells in the dentate gyrus of the rat hippocampal formation after acute VNS. (**a** and **b**) The number of $BrdU^+$ cells in the subgranular zone and granule cell layer of the dorsal dentate gyrus was determined 24 h (**a**) or 3 weeks (**b**) after administration of VNS for 3 h by multiplying by 6 the number of positive cells counted in 24 sections with a thickness of 16 μm and spaced 96 μm apart per rat. Data are means ± SEM of values from six rats per group. *P* values for comparison between VNS-treated and sham-operated animals were determined by ANOVA followed by Scheffe's test. (**c** and **d**) Representative bright-field images of immunohistochemical staining for both BrdU and NeuN in sections of the dorsal dentate gyrus obtained from rats 24 h (**c**) or 3 weeks (**d**) after acute VNS stimulation. Newly generated cells, the nuclei of which were stained dark blue for BrdU immunoreactivity (arrows), were detected in the subgranular zone in (**c**) and in the inner granule cell layer (stained red for NeuN immunoreactivity) in (**d**). Scale bars, 20 μm.

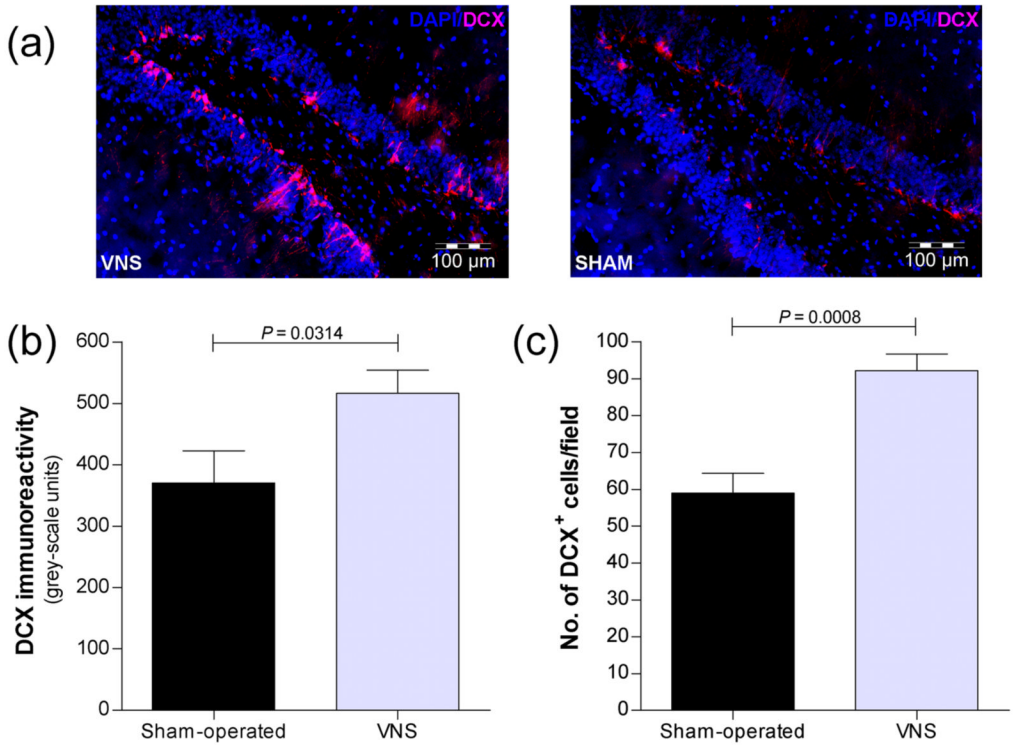

Figure 2.

Increase in the number of DCX^+ neurons in the dentate gyrus of the rat hippocampal formation induced by acute VNS. (**a**) Immunofluorescence staining for DCX (red) and nuclear staining with DAPI (blue) in the two blades of the granule cell layer of the dorsal dentate gyrus obtained from rats 3 weeks after acute VNS or from sham-operated controls. Scale bar, 100 µm. (**b** and **c**) Quantitation of DCX immunoreactivity (**b**) and the number of DCX^+ neurons per field (**c**) in the dorsal dentate gyrus of rats treated as in (**a**) was performed as described in Methods and Materials. Data are means ± SEM of values from six rats per group. *P* values for the indicated comparisons were determined by ANOVA followed by Scheffe's test.

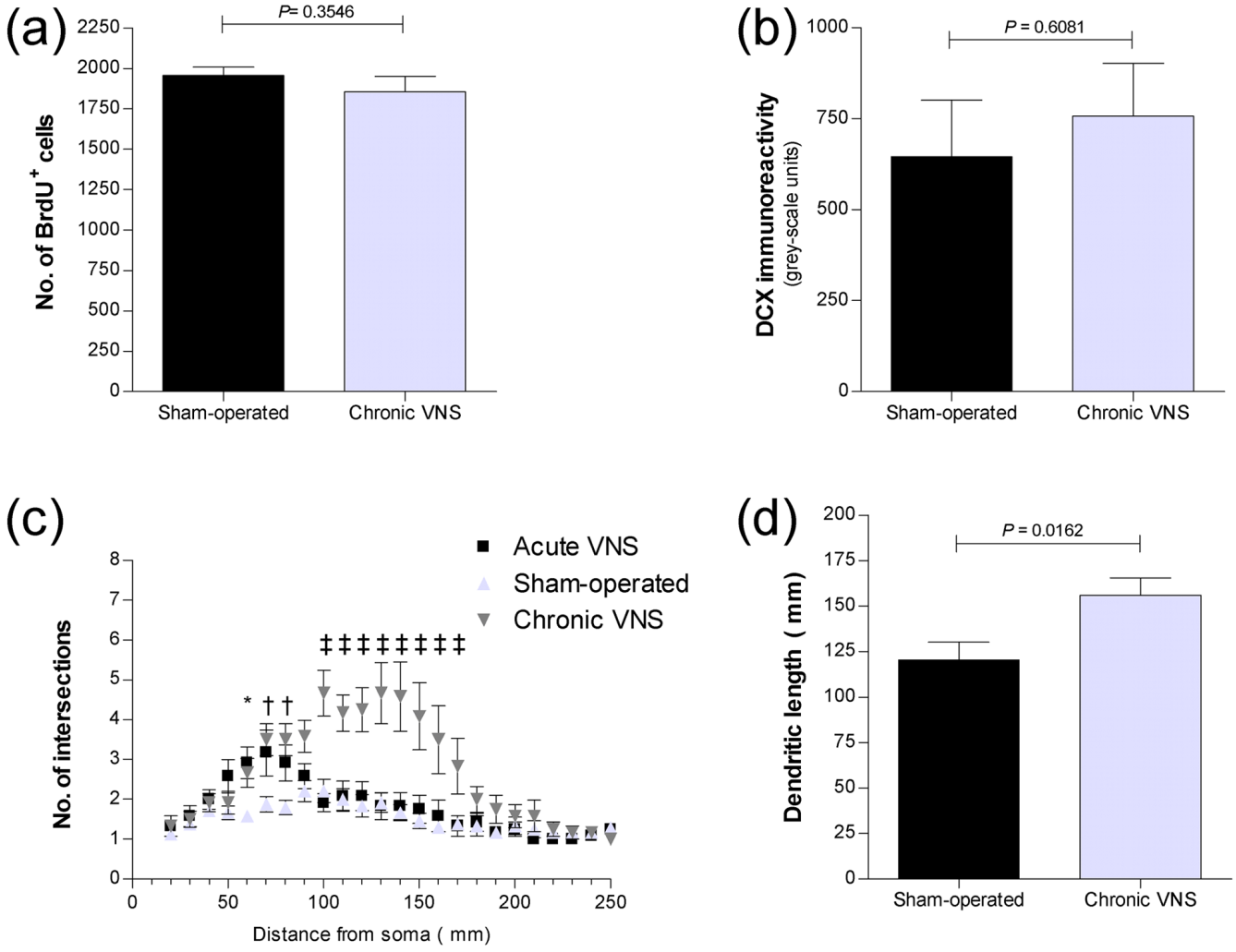

Figure 3.

Quantitation of newly generated cells (**a**) and effects of chronic VNS on the dendritic morphology of DCX^+ neurons (**b–f**) in the dentate gyrus of the rat hippocampal formation after chronic VNS. (**a**) The number of $BrdU^+$ cells in the subgranular zone and granule cell layer of the dorsal dentate gyrus was determined (as in Figure 1) 3 weeks after administration of VNS for 1 month. Data are means ± SEM of values from six rats per group. The *P* value for comparison between VNS-treated and sham-operated animals was determined by ANOVA followed by Scheffe's test. (**b**) Quantitation of DCX immunoreactivity in the dorsal dentate gyrus 3 weeks after chronic VNS for 1 month. Data are means ± SEM of values from six rats per group. The *P* value for comparison with sham-operated animals was determined by ANOVA followed by Scheffe's test. (**c**) Sholl analysis of apical dendrites of DCX^+ neurons in the dorsal dentate gyrus of rats 3 weeks after acute or chronic VNS. The numbers of dendrites that cross the indicated radial distances (0 to 250 μm) from the soma are shown. Data are means ± SEM of values from six rats per group. $*P < 0.05$, $\dagger P < 0.01$, $\ddagger P < 0.001$ versus corresponding sham-operated controls (Newman-Keuls test). (**d**) Dendritic length for DCX^+ neurons in the dorsal dentate gyrus of rats 3 weeks after chronic VNS. Data are means ± SEM of values from six rats per group. The *P* value for comparison with sham-operated controls was determined by ANOVA followed by Scheffe's test. (**e–f**) Representative immunofluorescence images of neurons positive for DCX or NeuN in the dentate gyrus of the rat hippocampal formation after

chronic VNS. Sections of the dorsal dentate gyrus obtained from rats 3 weeks after chronic VNS (**f**) or from sham-operated controls (**e**) were stained with antibodies to DCX (red) or to NeuN (green). The boxed regions in the top panels are shown at higher magnification in the bottom panels. Note the increase in dendritic complexity and length for DCX^+ neurons in rats subjected to VNS compared with those in control animals. The dendrites project deeply into the hippocampal molecular layer through the granule cell layer stained with the neuronal marker NeuN. Arrows in the merged images indicate that most DCX^+ neurons were also positive for NeuN.

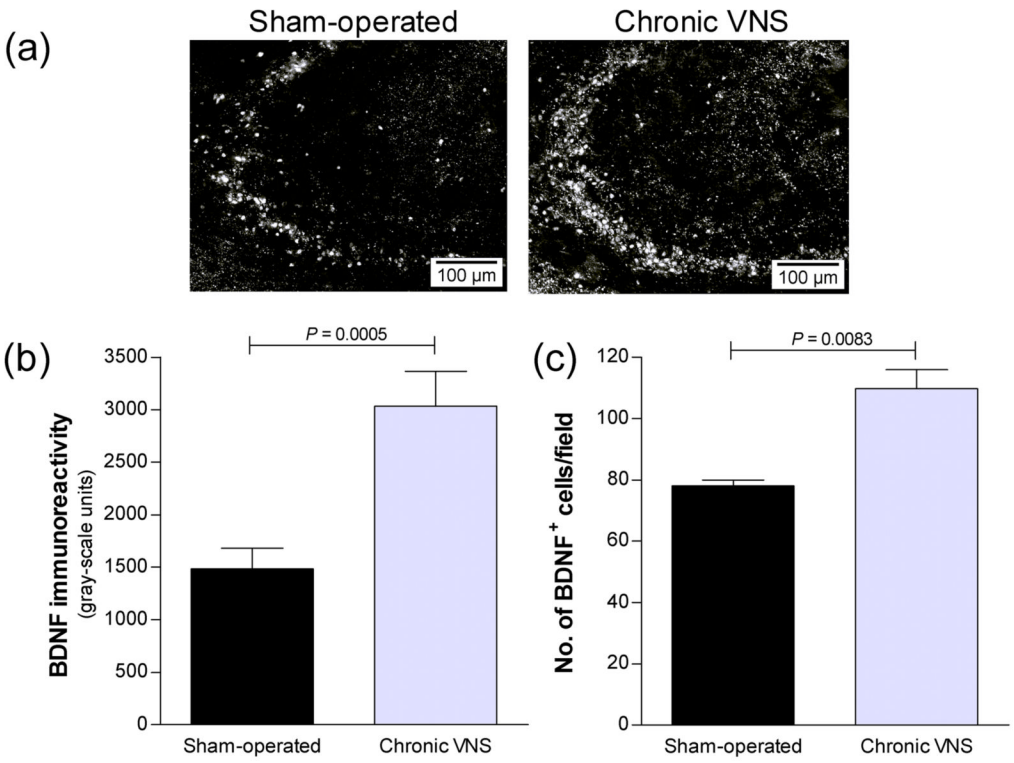

Figure 4.

Increase in BDNF expression in the CA3 region of the rat hippocampus after chronic VNS. (**a**) Sections of the CA3 region obtained from rats 3 weeks after chronic VNS or from sham-operated controls were subjected to immunofluorescence staining for BDNF. (**b** and **c**) Quantitation of BDNF immunoreactivity (**b**) and the number of $BDNF^+$ neurons per field (**c**) in the CA3 region of rats treated as in (**a**) was performed as described in Methods and Materials. Data are means ± SEM of values from six rats per group. *P* values for the indicated comparisons were determined by ANOVA followed by Scheffe's test.

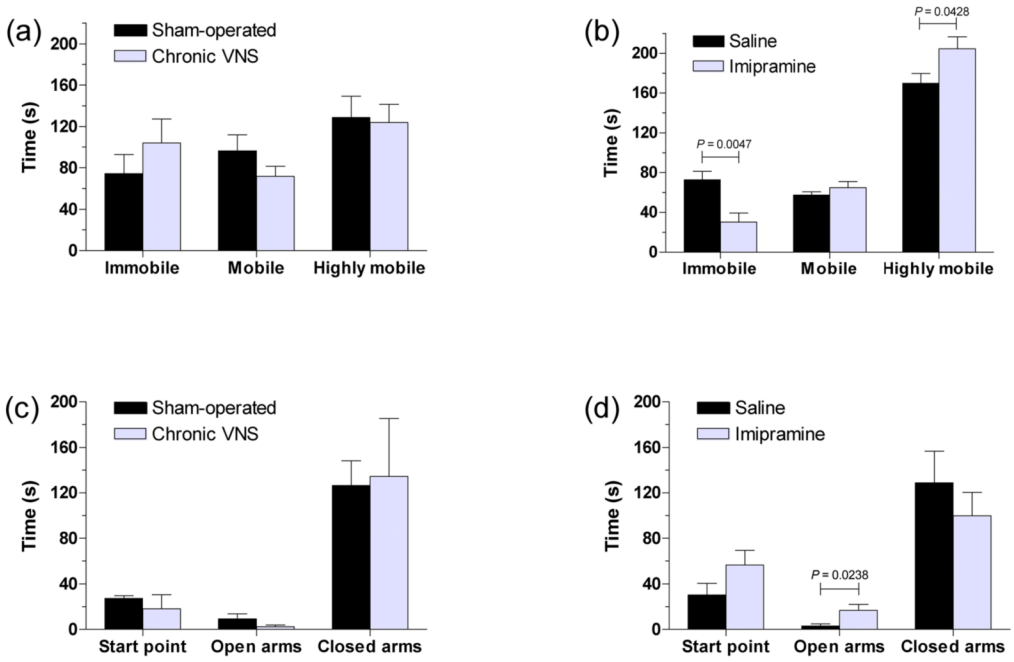

Figure 5.

Effects of chronic VNS on rat behavior in comparison with those of chronic imipramine treatment. (**a** and **b**) Rats were subjected to the forced swim test either the last day of chronic VNS with the stimulation device still on (**a**) or 30 min after the last injection of chronic imipramine treatment (**b**). Sham-operated or saline-treated animals were also examined as respective controls. Times of immobility, mobility, and high mobility were determined. (**c** and **d**) Rats were subjected to the elevated plus-maze test the last day of chronic VNS with the stimulation device still on (**c**) or 30 min after the last injection of chronic imipramine treatment (**d**). Data are means ± SEM from six VNS or sham-operated or ten imipramine or control rats. *$P < 0.05$, **$P < 0.01$ versus respective control animals (ANOVA followed by Scheffe's test).