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Vagus nerve stimulation ameliorated deficits in one-way active avoidance learning and stimulated hippocampal neurogenesis in bulbectomized rats

Nils Gebhardt [a], Karl-Jürgen Bär [b], Michael K. Boettger [c,1], Gisela Grecksch [a], Gerburg Keilhoff [d], Rupert Reichart [e], Axel Becker [a,*]

[a] O.-v.-Guericke University, Faculty of Medicine, Institute of Pharmacology and Toxicology, Leipziger Str. 44, D-39120 Magdeburg, Germany
[b] Pain & Autonomics – Integrative Research (PAIR), Department of Psychiatry and Psychotherapy, Philosophenweg 3, University Hospital, Jena, D-07747 Jena, Germany
[c] Institute of Physiology I, University Hospital Jena, Teichgraben 8, D-07743 Jena, Germany
[d] O.-v.-Guericke University, Institute of Biochemistry and Cell Biology, Leipziger Str. 44, D-39120 Magdeburg, Germany
[e] Department of Neurosurgery, Erlanger Allee 101, University Hospital, Jena, D-07747 Jena, Germany

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ABSTRACT

Background: Vagus nerve stimulation (VNS) has been introduced as a therapeutic option for treatment-resistant depression. The neural and chemical mechanisms responsible for the effects of VNS are largely unclear.
Methods: Bilateral removal of the olfactory bulbs (OBX) is a validated animal model in depression research. We studied the effects of vagus nerve stimulation (VNS) on disturbed one-way active avoidance learning and neurogenesis in the hippocampal dentate gyrus of rats.
Results: After a stimulation period of 3 weeks, OBX rats acquired the learning task as controls. In addition, the OBX-related decrease of neuronal differentiated BrdU positive cells in the dentate gyrus was prevented by VNS.
Conclusions: This suggests that chronic VNS and changes in hippocampal neurogenesis induced by VNS may also account for the amelioration of behavioral deficits in OBX rats. To the best of our knowledge, this is the first report on the restorative effects of VNS on behavioral function in an animal model of depression that can be compared with the effects of antidepressants.

Introduction

Depression is a common and often debilitating disorder. There are clear limitations to the currently approved pharmacotherapies of depression. These include modest efficacy, a relatively slow onset of beneficial effects and significant side effects such as sexual dysfunction, weight gain or cognitive impairment. In addition, incomplete remission of depressive symptoms is commonly observed. It was reported that about one-third of patients treated for major depressive disorder (MDD), do not respond satisfactorily to the first antidepressant, and up to 15% of cases remain significantly depressed despite multiple pharmacological and psychotherapeutic approaches [1–6]. Thus, new concepts in the treatment of depression aimed at achieving a sustained response and the remission of depressive symptoms are needed.

Vagus nerve stimulation (VNS), which is one of the new electrical stimulation therapies which has been introduced as a therapeutic option for treatment-resistant depression [7–10]. Although clinical trials for treatment-resistant depression (TRD) showed variable clinical response rates after 10 weeks, most encouragingly, patients continued to improve after the acute phase for up to one year [11]. Despite the growing amount of clinical data, the neural and chemical mechanisms responsible for the effects of VNS are largely unclear. Animal experiments have shown that VNS triggers neurochemical and molecular changes in the rat brain [12] and induces neuronal plasticity in the rat hippocampus [13,14]. In addition, the behavioral effects of VNS stimulation in an animal model of depression have not yet been shown. An accepted and widely used animal model in depression research is the bilateral removal of the olfactory bulbs (OBX) [15–18]. In OBX rats, specific alterations in the parameters of 5-hydroxytryptaminergic presynaptic neurotransmission were described. In detail, a serotonergic hyperinnervation of the frontal cortex was found [19]. After subchronic treatment with the tricyclic antidepressant imipramine, OBX rats acquired the learning task as controls. This was explained in terms of modulation

* Corresponding author. Tel.: +49 391 6715351; fax: +49 391 67290149.
E-mail address: axel.becker@med.ovgu.de (A. Becker).
[1] Present address: Bayer Pharma AG, Forschungszentrum Elberfeld, Aprather Weg 18a, D-42113 Wuppertal, Germany.

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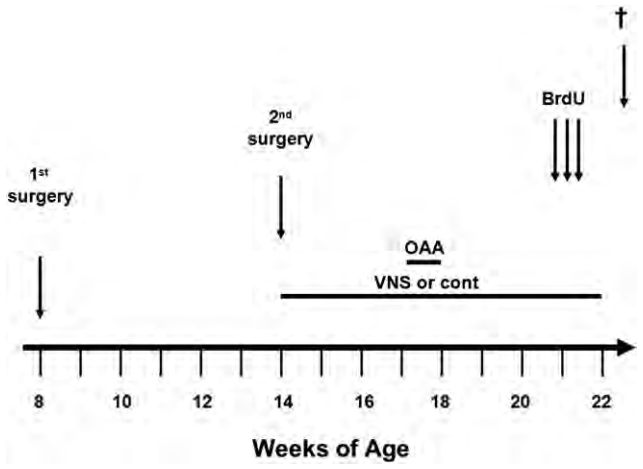

Figure 1. Experimental schedule. 1st surgery bulbectomy or sham bulbectomy, 2nd surgery implantation of stimulation device or dummy, VNS vagus nerve stimulation or control, OAA one-way active avoidance learning, BrdU injection, †collection of the brains.

of parameters in the serotonergic neurotransmission system. Due to antidepressant treatment, the increased output of the 5-HT projections in the frontal cortex was attenuated through the destabilization of the rate limiting enzyme of 5-HT synthesis [20]. Moreover, these rats showed a significant impairment in learning a conditioned reaction in an active one-way avoidance task (pole jumping). Both the neurochemical alterations and the learning impairment were normalized after chronic treatment with the tricyclic antidepressant imipramine [19,20]. Interestingly enough, cell proliferation in the dentate gyrus was found to be influenced by OBX and was normalized by imipramine treatment in a region-specific manner [21]. This suggests that OBX is a useful model for the investigation of VNS antidepressant effects and their underlying neurobiological mechanisms. Our study set out to investigate whether the antidepressive effects of VNS seen in humans might be observable in an animal model of depression, particularly focusing on behavioral aspects. A successful approach would allow the translation of clinical effects into an animal model to understand and investigate basic mechanisms, and to identify key issues for later clinical application. In addition, it would further validate the animal model of OBX with respect to this kind of translational research. Therefore, we used a one-way active avoidance learning task as a correlate of cognition as a behavioral surrogate parameter of depression-associated alterations and studied cell proliferation in the dentate gyrus in OBX and control rats.

Material and methods

The work reported here was conducted in accordance with EC regulations and those of the National Act on the Use of Experimental Animals (Germany). The protocol was approved by the Saxony-Anhalt Committee on Animal Care. Fig. 1 shows the sequence of all the experiments which were performed.

Animals

The animals used were male Wistar rats (Shoe:Wist(Shoe), DIMED Schönwalde GmbH). The rats were kept under controlled laboratory conditions with a light/dark cycle 12:12 (lights on at 6 a.m.), temperature 20 ± 2 °C, and air humidity 55–60%. The animals had free access to commercial rat pellets (ssniff R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany) and tap water. The animals were housed in groups of 5 in Macrolon IV cages, and after implantation of the VNS device rats were housed singly in Macrolon III cages. OBX or sham surgery (OBS) was performed on 8 week old rats.

In total, 40 animals were used. At the end of the experiment, each animal was examined post mortem to ensure that the olfactory bulbs had been completely removed and there was no damage to the frontal cortex. Only such animals were included in the results and for statistical evaluation (37 out of 40).

Surgery

Bilateral olfactory bulbectomy (OBX) was performed as described by O'Connor and Leonard [22]. Briefly, rats were deeply anesthetized with pentobarbital (40 mg/kg body weight, intraperitoneally i.p., injection volume 10 ml/kg body weight) and a midline skin incision was made to expose the skull overlying the bulbs. Two holes (diameter 2 mm) were drilled above the bulbs (6.5 mm anterior to the bregma, 2 mm laterally on both sides of the midline). The olfactory bulbs were cut and removed by aspiration using a deflected pipette. The resulting space was filled with hemostatic sponges (Gelitaspon®, Gelida Medical, Amsterdam, The Netherlands), and the skin was closed with tissue adhesive (Histoacryl®, B. Braun Aesculap, Tuttlingen, Germany). Sham-operated rats (OBS) were treated in the same manner, including piercing of the dura, but the bulbs were left intact.

Six weeks after OBX or OBS surgery, stimulators and electrodes for VNS were implanted according to the method described by Follesa et al. [12]. Under deep pentobarbital anesthesia (see above), an incision was made at the left side of the ventral neck, and the vagus nerve was isolated. The bipolar electrodes were carefully wrapped around the nerve. The electrode cable was tunneled towards a horizontal incision on the right flank. A pocket was formed for the pulse generator (Cyberonics 102, Cyberonics, Inc., Houston, TX, USA) and they were connected to the pulse generator. A control group of animals was subjected to the same surgery with electrodes connected to a dummy pulse generator equal in size and weight to the working one. The muscular layer was closed with 4/0 resorbable sutures and the skin incision was closed with 3/0 silk. Two days after surgery, the pulse generator was activated. Stimulation parameters were chosen according to Bohotin et al. [23]: 2 mA, 30 Hz, pulse width 500 μs, 30 s on, 300 s off. After a three-week period of continuous stimulation, the pole jumping experiment was performed.

Pole jumping

The learning experiment was performed when the rats were aged 17 weeks (8 weeks at OBX/OBS + 6 weeks for recovery + 3 weeks for stimulation). The pole jumping apparatus (30 × 30 × 50 cm) was situated in a sound-proofed room. Rats were trained to jump onto a pole (1.5 cm in diameter) during a 4 s presentation of a sound signal (80 dB) produced by a buzzer (conditioned stimulus, CS) in order to avoid electric foot shock (unconditioned stimulus, UCS) delivered through stainless steel rods forming the floor. Electric foot shock (0.2–0.4 mA) was adjusted to the rat's individual pain sensitivity and was at about the audible vocalization threshold. The CS and UCS were switched off when the animal jumped onto the pole. The maximum trial duration was 20 s. The duration of inter-trial intervals was randomized, with a mean inter-trial interval of 60 s (30–90 s). Ten trials were performed daily on 5 consecutive days. Prior to the first session, the rats were allowed to explore the box for 5 min and on the following days 1 min was provided. Sessions were performed during the light part of the daily cycle at about the same time ±1 h (8:00 a.m.–2:00 pm). The numbers of conditioned (reaction time < 4 s) and

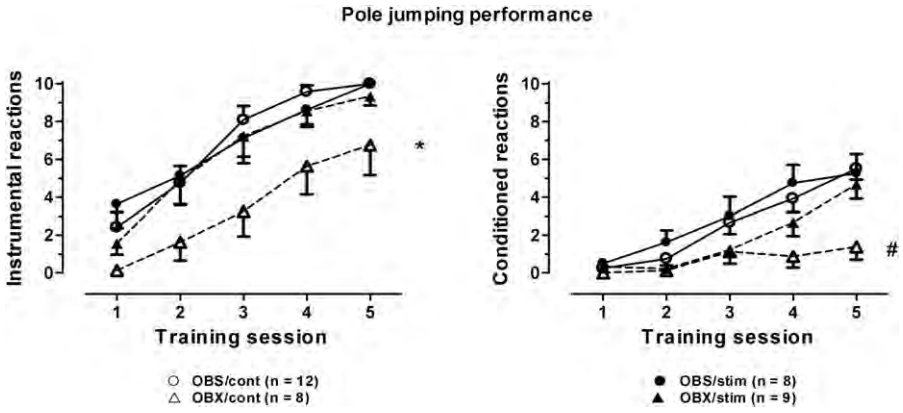

Figure 2. Effect of vagus nerve stimulation on one-way active avoidance learning (pole jumping) in bulbectomized (OBX) and sham-operated (OBS) rats. Six weeks after surgery, rats were subjected to electrical stimulation of the vagus nerve (stim). Control animals (cont) were implanted with a dummy stimulator. OBX/cont rats acquired the instrumental reaction in a similar way to the other groups, but the number of instrumental reactions was significantly lower (*$P < 0.05$). The acquisition and number of conditioned reactions is significantly different (#$P < 0.05$) in the OBX/cont group. n = number of animals per group. Mean ± SEM.

instrumental reactions (=conditioned + unconditioned reactions, reaction time 0–20 s) were recorded for further evaluation.

During the learning experiment, the pulse generator was switched off.

Assessment of cell proliferation

An immunofluorescence assay was performed for the detection of 5-bromo-2′-deoxy-uridine (BrdU, Boehringer Mannheim, Germany) incorporated into cellular DNA. Rats received one daily intraperitoneal (i.p.) injection of 50 mg/kg BrdU dissolved in physiological saline on three consecutive days; this began three weeks after the learning experiment. Fifty mg/kg (i.p.) is a common dose in rodents, offering comparability with other published results.

Seven days after the final BrdU injection (i.e. after a total stimulation period of 8 weeks), anesthetized rats (isofluran, Baxter, Germany) were sacrificed by transcardial perfusion (4% 0.1 M phosphate-buffered paraformaldehyde, Merck, Darmstadt, Germany, pH 7.4), and post-fixed in the same fixative overnight at room temperature, cryoprotected for 2 days in a solution of 30% sucrose (Merck) in 0.4% buffered paraformaldehyde (pH 7.4) and rapidly frozen to minus 20 °C using 2-methylbutane (Roth, Karlsruhe, Germany). Free-floating serial sagittal sections (20 μm) were cut on a cryostat (Jung Frigocut 2800 E, Leica, Bensheim, Germany), and immunolabeled. For details of the staining procedure, see Keilhoff et al. [21]. In brief: pre-incubation of cryo-sections in 2 M HCl, neutralized with 0.1 M borate buffer; incubation with a rat monoclonal antibody to BrdU (Serotec, Oxford, UK, 1:100) in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 (Merck, Darmstadt, Germany) for 1 h at 37 °C; several rinses in PBS; incubation with a monoclonal mouse anti-NeuN antibody (Chemicon, Billerica, MA, USA, 1:100), or a polyclonal rabbit anti-β-III-Tubulin antibody (Covance, Princeton, NJ, USA, 1:1000) in PBS with 0.3% Triton X-100 and 1% normal goat serum for 3 h at 37 °C (for cell characterization); incubation with a combination of secondary antibodies (Molecular Probes, Göttingen, Germany, 1:500): goat anti-rat-IgG Alexa Fluor 546/goat anti-mouse-IgG Alexa Fluor 488 or goat anti-rat-IgG Alexa Fluor 546/goat anti-rabbit-IgG Alexa Fluor 488; embedding; examination using a fluorescence microscope (Axio Imager.M1, Zeiss, Jena, Germany) equipped with fluorescein/rhodamine Plan-Neofluar objective (×40/0.75). The control experiment (substitution of the primary antiserum with PBS) yielded no specific immunostaining.

Cell quantification was done according to a standard protocol. The investigator, blinded to the experimental protocol, counted all cell profiles with clear immuno-staining in the hippocampal dentate gyrus. Thus for each animal, 4 sections were scanned image field by image field using the Axio Imager microscope with a Plan-Neofluar objective (×40/0.75). The AxioVision 'Panorama' software (Zeiss, Jena, Germany) generated a large composite image from the individual images representing the complete dentate gyrus. Two optical planes at the upper and lower slice surface were defined with the help of the AxioVision z-stack software (Zeiss, Jena, Germany). The stack was merged to one image with information gathered from all the labeled cells in focus. Data per animal represents the mean of the 4 scanned slices related to the complete dentate gyrus per slice.

Statistics

Learning performance was analyzed by repeated-measures ANOVA with the between subjects factor 'group' (OBX versus OBS) and the within subjects factor 'time' (examination days). To evaluate differences in the number of BrdU positive cells as well as β-III Tubulin and NeuN double-stained cells, a two-way ANOVA with the factors 'group' (OBX vs. OBS) and 'treatment' (stimulator, stim, and dummy, cont) was performed, followed by post-hoc Bonferroni tests. The significance threshold was set at 0.05. All data is presented as means ± SEM.

Results

Pole jumping instrumental reaction

Compared with other experiments performed in our laboratory [20], the learning performance of all animals is at a moderate level (Fig. 2). This might be a consequence of the weight of the stimulation device.

As shown in Fig. 2, the animals learned the instrumental reaction regardless of surgery. Within the period of 5 days, the number of instrumental reactions was constantly increasing and there were no significant differences between the groups (surgery F 4, 132 = 0.15, $P = 0.06$, treatment F 4, 132 = 0.21, $P = 0.93$). However, if one considers the mean group number of instrumental reactions, there was a significant difference F 1, 3 = 4.83, $P = 0.007$. OBX/cont had significantly fewer instrumental reactions compared to OBS/cont ($P = 0.008$) and OBS/stim ($P = 0.02$). The difference between OBX/cont and OBX/stim did not reach the significance threshold ($P = 0.07$).

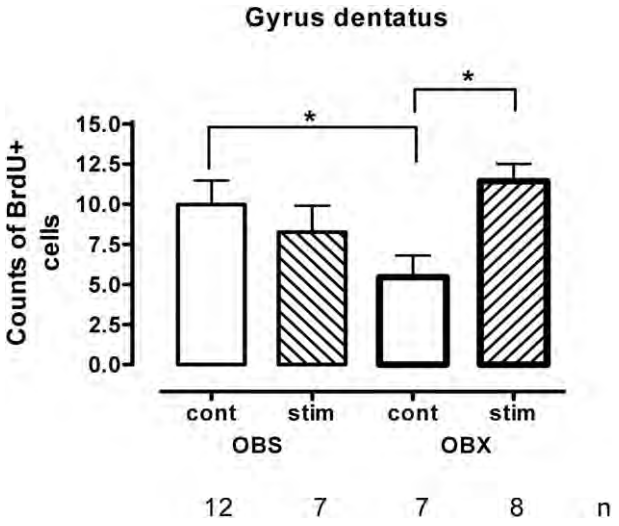

Figure 3. Quantitative analysis of BrdU positive cells in the dentate gyrus in the hippocampus of rats after bilateral removal of the olfactory bulbs (OBX) or sham surgery (OBS). Six weeks after surgery, rats were subjected to electrical stimulation of the vagus nerve (stim). Control animals (cont) were only implanted with a dummy stimulator. $^*P < 0.05$. n = number of animals per group. Mean ± SEM.

Pole jumping conditioned reaction

Within the test period of 5 days, we found a significant effect of surgery F 4, 132 = 2.79, P = 0.03, treatment F 4, 132 = 3.85, P = 0.006, and a significant surgery × treatment interaction F 4, 132 = 6.26, $P < 0.001$. Exceptionally in the OBX/cont group, the number of conditioned reactions increased constantly (Fig. 2). The total number of conditioned reactions in the OBX/cont groups was significantly lower compared with the other groups ($P < 0.05$). This indicates that stimulation had a restorative effect on learning the conditioned reactions in OBX animals. OBX/stim animals attained a similar number of conditioned reactions as OBS/cont.

Cell proliferation

There was a significant difference between the 4 experimental groups (F 3, 33 = 3.92, P = 0.018) and a significant surgery × treatment interaction (F 3, 33 = 9.07, P = 0.005). Post hoc analysis revealed a significant reduction in the number of BrdU positive cells in OBX/cont rats (vs. OBS/cont P = 0.04) by about 50% in the dentate gyrus. Interestingly, this reduction was prevented altogether by VNS. The number of BrdU positive cells in the dentate gyrus of OBX/stim animals was significantly higher compared with OBX/cont rats (P = 0.024), and there was no difference in the number of BrdU positive cells between OBS/cont and OBX/stim rats (Fig. 3). In the OBS group, stimulation had no effect (OBS/cont vs. OBS/stim P = 1.0). Representative slices demonstrated mitotic activity in the dentate gyrus of rats after OBS or OBX with or without VNS (Fig. 4A–D). Double staining for BrdU and β-III-Tubulin (Fig. 4E–H) and NeuN (Fig. 4I–L) revealed that the majority of "newcomers" were neuronal (~60% β-III-Tubulin+, ~5% NeuN+, Table 1), whereby the cell type pattern is stable and treatment independent. Two-way ANOVA revealed no significant differences between the groups and there were no interactions (β-III-Tubulin surgery F 3, 19 = 1.64, P = 0.22, treatment F 3, 19 = 4.11, P = 0.53,

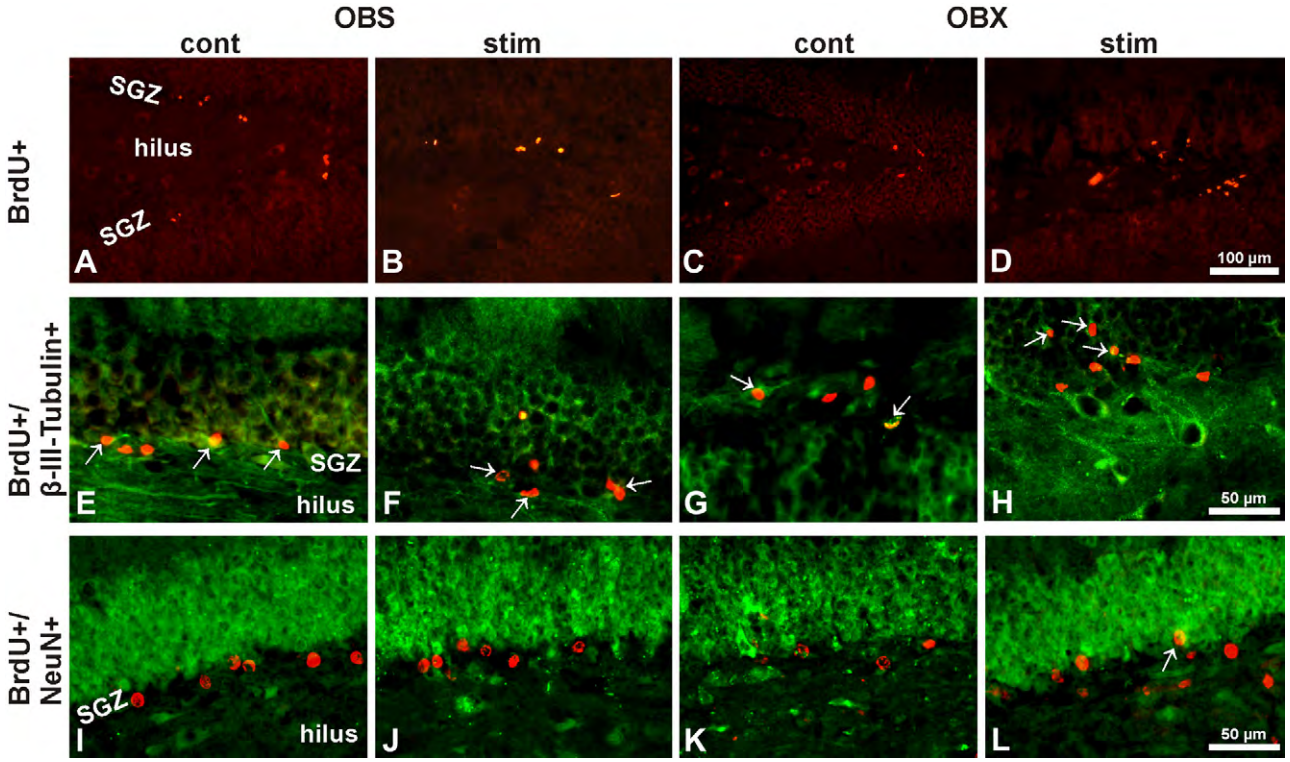

Figure 4. Representative slices showing immunohistochemical: BrdU-labeling (A–D) of nuclei (orange dots) in the dentate gyrus of rats after bilateral removal of the olfactory bulbs (OBX) or sham surgery (OBS) with (stim) or without (cont) electrical stimulation of the vagus nerve (VNS). Baseline mitotic activity (A) is unchanged after VNS in sham-operated animals (B), significantly decreased after bulbectomy (C), and normalized by VNS after bulbectomy (D); (E–H) BrdU (red)/β-III-Tubulin (green) co-staining (yellow-orange, arrows), indicating a lot of newly formed cells as early neuronal stages; (I–L) BrdU (red)/NeuN (green) co-staining (yellow-orange, arrow in L), indicating that one week after BrdU-application majority of newcomers are not yet mature neurons. Per group 5 animals were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number (Mean ± SEM) of double stained (BrdU, β-III-Tubulin and NeuN, respectively) in the dentate gyrus of rats after bilateral removal of the olfactory bulbs (OBX) or sham surgery (OBS) after stimulation (stim) or without stimulation (cont). Per group 5 animals were used.

	OBS		OBX	
	cont	stim	cont	stim
B-III-Tubulin	62.4 ± 8.87	61.45 ± 9.82	57.1 ± 7.41	60.95 ± 9.03
NeuN	5.2 ± 1.47	5.4 ± 1.02	4.95 ± 1.66	5.3 ± 1.42

surgery × treatment interaction $F\,1, 19 = 1.12$, $P = 0.3$, NeuN surgery $F\ 3,\ 19 = 0.5$, $P = 0.49$, treatment $F\ 3,\ 19 = 1.19$, $P = 0.29$, surgery × treatment interaction $F\,1, 19 = 0.088$, $P = 0.77$).

Discussion

In this study, we describe the beneficial effects of VNS on behavioral correlates of cognition in an animal model of depression and on neuromorphological alterations associated with depression.

Clinical trials have shown that VNS is a useful non-pharmacological option in the treatment of severe depression and TRD. An ever-increasing body of clinical data is shedding light on the mechanisms underlying the therapeutic effects of VNS [10,24–27]. However, molecular mechanisms underlying changes in the serotonergic system or relating to neuroplasticity cannot be derived from clinical data, so there is a need for experimental preclinical data to help us understand this therapeutic option in detail.

We used OBX rats to investigate the effects of VNS, which is considered a valid animal model in depression research. In former studies it was shown that the acquisition of a one-way active avoidance task is impaired in these animals. Interestingly, after 3 weeks of continuous VNS we found an equivalent effect on one-way active avoidance learning equivalent to treatment with the tricyclic antidepressant imipramine [20] (Fig. 2). To the best of our knowledge, this is the first report demonstrating that VNS ameliorated learning impairment in an animal model of depression. VNS was shown to modulate the concentration of different neurotransmitters [10]. Obviously, these changes are region-specific in the brain. No significant changes could be demonstrated for GABA, 5-HT and dopamine, but a significant increase in hippocampal noradrenaline levels was induced [28]. In another study, it was found that VNS treatment induces large time-dependent increases in basal neuronal firing in the dorsal raphe nucleus and locus coeruleus for serotonin and noradrenaline [29]. The authors concluded that the antidepressant effects of VNS are due to increased firing activity of both serotonergic and noradrenergic neurones. They proposed this as a new mechanism of action of antidepressants, which explains the beneficial effects for patients with TRD. Assuming that the VNS protocol employed in our study induced similar alterations in the parameters of serotonergic and noradrenergic neurotransmission leading to antidepressant effects, this would explain the improved one-way active avoidance learning in OBX rats (Fig. 2).

Clinical [30–32] and experimental [33–36] studies have shown that neurogenesis is altered in depression and that treatment with antidepressants normalizes these alterations at least in part. In OBX rats, neurogenesis was reduced and this effect was ameliorated by treatment with the tricyclic antidepressant imipramine [21]. A relationship between depression and neurogenesis has also been described by others [37,38], and intact neurogenesis was suggested as a precondition for the therapeutic effects of antidepressants [39]. In normal rats, VNS also induced neurogenesis [13,14]. This effect occurred even after short-term stimulation and was dependent on stimulus intensity. The relationship between stimulus intensity and effect was found to be inverted U-shaped. Moreover, VNS increased the expression of trophic factors such as BDNF and bFGF in specific brain regions [12]. It was suggested that VNS induces molecular and cellular effects contributing to functional changes associated with neuronal plasticity as described after treatment with clinically used antidepressants [40,41].

The present experiments confirm previously published data that the number of BrdU positive cells in the dentate gyrus is lower in OBX rats (Figs. 3 and 4). Interestingly, after a stimulation period of 8 weeks there was no difference between sham-operated controls and stimulated OBX rats in this parameter, indicating significant restorative effects of VNS in our model. In OBS/stim rats, no increased number of BrdU positive cells was found. The discrepancy with other results demonstrating neurogenesis in normal rats [13] might be a consequence of different stimulation protocols with particular regard to the duration of the stimulation period. VNS leads to acute changes in different brain structures, whereas chronic VNS results in significant ventromedial prefrontal cortex deactivation, similarly to other approaches in the treatment of depression [42]. It is particularly interesting that increased neurogenesis was detectable in stimulated OBX rats only. If the stimulation protocol used leads to similar effects on the expression of trophic factors, this could explain the resulting antidepressant effect. Moreover, it would also explain the restorative effect of VNS on one-way active avoidance learning, because these factors also play a crucial role in processes related to learning and memory formation [43–46].

In conclusion, we described a normalization of depression-related behavior in the olfactory bulbectomy rat model by VNS, which is similar to effects seen with pharmacological intervention.

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