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Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model

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Abstract

Vagus nerve stimulation (VNS) is an effective adjunctive treatment for medically refractory epilepsy. In this study, we measured VNS-induced changes in hippocampal neurotransmitter levels and determined their potential involvement in the anticonvulsive action of VNS, to elucidate the mechanism of action responsible for the seizure suppressing effect of VNS in an animal model for limbic seizures. We used *in vivo* intracerebral microdialysis to measure VNS-induced changes in hippocampal extracellular concentrations of noradrenaline, dopamine, serotonin and GABA in freely moving, male Wistar rats. During the same experiment, the effect of VNS on pilocarpine-induced limbic seizures was assessed using video-EEG monitoring. The involvement of VNS-induced increases in hippocampal noradrenaline in the mechanims of action of VNS was evaluated by blocking hippocampal α_2-receptors. VNS produced a significant increase in hippocampal noradrenaline concentration (69 ± 16% above baseline levels). VNS also increased the latency between pilocarpine infusion and the onset of epileptiform discharges, and reduced the duration and severity of pilocarpine-induced limbic seizures. A strong positive correlation was found between the noradrenergic and anticonvulsive effects of VNS. Blockade of hippocampal α_2-receptors reversed the seizure-suppressing effect of VNS. VNS induces increases in extracellular hippocampal noradrenaline, which are at least partly responsible for its seizure-suppressing effect in a model for limbic seizures, and constitute a potential biomarker for the efficacy of VNS in temporal lobe epilepsy.

Keywords: EEG, Hippocampus, noradrenaline, pilocarpine, temporal lobe epilepsy, vagus nerve stimulation.

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Vagus nerve stimulation (VNS) is an adjunctive treatment for refractory epilepsy in patients who are unsuitable candidates for epilepsy surgery (Ben-Menachem 2002). Worldwide, more than 50 000 epilepsy patients have been treated with VNS. Several studies, including two large double-blind randomized clinical trials (Ben-Menachem *et al.* 1994; DeGiorgio *et al.* 2000), have confirmed the efficacy of VNS in different types of epilepsy. Seizure reduction as a result of VNS ranges from 25% to 55%, and varies considerably from patient to patient. In responders, VNS causes either a rapid or a delayed reduction in seizure frequency. However, a significant fraction (approximately one third) of patients do not respond to VNS. Because the mechanism of action of VNS in epilepsy is currently unknown, it is not clear which factors determine the patient's response to the treatment, nor what the most optimal stimulation parameters are.

The vagus nerve is a mixed nerve consisting of 20% efferent (motor) and 80% afferent (sensory) fibers. The nucleus of the solitary tract receives the largest number of vagal afferents. The nucleus of the solitary tract in turn

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Abbreviations used: LC, locus coeruleus; SSS, seizure severity score; TLE, temporal lobe epilepsy; TSSS, total SSS; VNS, vagus nerve stimulation.

projects to pontine nuclei, the cerebellum and the mesencephalon, but also to regions which are frequently involved in the generation of seizures such as the cortex, thalamus and amygdala. The vagus nerve also projects directly to the raphe nucleus and indirectly to the locus coeruleus (LC). These nuclei are the major sources of serotonergic and noradrenergic neurons in the brain, respectively (Henry 2002). Both send direct projections to the hippocampus, a brain structure that is frequently involved in the generation of epileptic seizures in temporal lobe epilepsy (TLE) (Castle *et al.* 2005).

Interestingly, bilateral destruction of the LC has been found to reverse the seizure-suppressing effect of VNS in the maximal electroshock model (Krahl *et al.* 1998). Single-unit recording experiments have shown that the activity of noradrenergic neurons in the LC is increased upon stimulation of the vagus nerve (Groves *et al.* 2005; Dorr and Debonnel 2006). Presumably as a result of enhancement of the activity of LC neurons, increases in extracellular noradrenaline concentration have been measured in projection areas of the LC such as the hippocampus and cortex in VNS-treated rats (Roosevelt *et al.* 2006).

The noradrenergic system has been convincingly implicated in the control of seizure activity, especially for seizures that spread along the limbic system (Weinshenker and Szot 2002a; Giorgi *et al.* 2004). Animals in which the noradrenergic system has been damaged (McIntyre and Edson 1981; Bortolotto and Cavalheiro 1986; Corcoran 1988) or is genetically impaired (Mishra *et al.* 1993; Yan *et al.* 1998; Szot *et al.* 1999) generally have a higher susceptibility to experimentally evoked seizures and seizure susceptibility in these models can be reduced by restoring noradrenergic activity (Gross and Ferrendelli 1982; Barry *et al.* 1987; ; Kokaia *et al.* 1989, 1994; Bengzon *et al.* 1990; Mishra *et al.* 1993; Yan *et al.* 1998). Loss of noradrenaline also attenuates the efficacy of a number of anticonvulsant therapies including the ketogenic diet and valproic acid (Schank *et al.* 2005; Weinshenker 2008). Conversely, activation of the noradrenergic system can inhibit seizure activity induced by electrical stimulation (McIntyre *et al.* 1982) or administration of chemoconvulsants (Ferraro *et al.* 1994). Noradrenaline interacts with three families of G-protein coupled adrenergic receptors (α_1, α_2, β receptors) which are further divided into different subtypes ($\alpha_{1A\text{-}B}$, α_{1D}, $\alpha_{2A\text{-}C}$, $\beta_{1\text{-}3}$ receptors) (Ramos and Arnsten 2007). It has highest affinity for α_2 adrenergic receptors, which are generally coupled to G_i proteins. The α_2 adrenergic receptor subtype is considered to be the most promiscous anticonvulsant adrenoreceptor (Weinshenker and Szot 2002).

Recent work by our group provides further insight into the relative contribution of different adrenergic receptor subtypes to the anticonvulsive action of noradrenaline. Administation of maprotiline, a selective noradrenaline reuptake inhibitor, in the intrahippocampal pilocarpine model for limbic seizures resulted in increased levels of brain noradrenaline and potent suppression of pilocarpine-induced limbic seizures. Administration of a threshold anticonvulsive dose of maprotiline resulted in a 70% increase of hippocampal noradrenaline levels. Using selective agonists and antagonists for the different adrenergic receptor subtypes, we then showed that seizure suppression was mediated by combined activation of α_2- and β_2-adrenergic receptors. Application of an antagonist of either α_2- (SKF-86466) or β_2-adrenergic receptors (ICI-118551) reversed the anticonvulsive effect of increased hippocampal noradrenaline. On the other hand, combined application of an α_2- (medetomidine) and β_2-adrenoreceptor agonist (salmeterol) was necessary to obtain seizures suppression (Clinckers *et al.* 2010).

Given the established role of increased noradrenergic transmission in the control of limbic seizures, it is conceivable that VNS produces its anticonvulsive effect by increasing noradrenaline levels in structures that are critically involved in the generation of limbic seizures, such as the hippocampus. To test this hypothesis would require that the noradrenergic and anticonvulsive effects of VNS are measured concomitantly in the same group of animals. This experiment has not been performed to date.

The aim of this study was to evaluate whether VNS-induced changes in hippocampal neurotransmitter levels, particularly those of noradrenaline, are involved in the mechanism of action of VNS in TLE. In a first series of experiments, we determined the effect of VNS on hippocampal noradrenaline, dopamine, serotonin and GABA levels and on pilocarpine-induced limbic seizures in the same group of animals. Subsequently, we tested the hypothesis that increases in hippocampal noradrenaline mediate the anticonvulsive effect of VNS. To this end, we studied the effect of blockade of hippocampal adrenoreceptors on the seizure suppressing action of VNS. We chose to use a selective α_2-adrenoreceptor antagonist in these experiments, given that hippocampal α_2-adrenoreceptor blockade was previously found to be sufficient to completely reverse the anticonvulsive effect of a noradrenaline reuptake inhibitor in the same animal model (Clinckers *et al.* 2010).

Methods

Chemicals and reagents

Pilocarpine HCl and SKF-86466 HCl were purchased from Sigma (St Louis, MO, USA). All other chemicals were analytical reagent grade or better and were obtained from Merck (Darmstadt, Germany). Aqueous solutions were made with purified water (Seralpur pro 90 CN, Belgolabo, Overijse, Belgium) and filtered through a 0.2 μm membrane filter. The aqueous perfusion solution for the microdialysis experiments, subsequently referred to as modified Ringer's solution, consisted of 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl. An antioxidant solution containing 3.3 mM L-cystein, 0.27 mM Na_2EDTA, 12.5 μM ascorbic acid and 100 mM glacial acetic acid was used to stabilize collected monoamines in the

dialysates. All compounds were dissolved in modified Ringer's solution and administered via the microdialysis probe.

Animals

Thirty-seven male Wistar rats (Charles River Laboratories, Brussels, Belgium) weighing 250–275 g were used. Animals were treated according to guidelines approved by the European Ethics Committee (decree 86/609/EEC). The study protocol was approved by the Animal Experimental Ethical Committee of Ghent University Hospital (ECP 08/47). All animals were kept under environmentally controlled conditions (12 h light/dark cycles, 20–23°C and 50% relative humidity) with food and water intake *ad libitum*.

Surgery

Rats were anesthetized with isoflurane (induction: 5%; maintenance:1–2%) and implanted with two epidural recording electrodes in the right and left os frontale, a ground (reference) electrode close to the sutura lambdoidea and four anchor screws bilaterally in the os frontale and parietale. A bipolar depth electrode was fixed to a microdialysis guide cannula (CMA/Microdialysis; Solna, Sweden), and both were stereotactically implanted in the left hippocampus (coordinates relative to bregma: rostro-caudal −5.6 mm; mediolateral −4.6 mm; dorso-ventral −4.6 mm, that is, 3 mm above final microdialysis probe membrane position). A custom-made silicone spiral cuff-electrode with platinum contacts was implanted around the left vagus nerve. To minimize post-operative pain, buprenorphine (1 mg/kg) was intraperitonally administrated and a 2% xylocaine gel was applied to the incision wounds. Animals were allowed to recover from surgery under an infrared lamp. Correct positioning of the microdialysis probe in the left hippocampus and the cuff electrode around the left vagus nerve was verified postmortem. Animals with aberrant probe or cuff electrode localization were excluded from the study.

Video-EEG monitoring and intracerebral microdialysis

One week after surgery, rats were placed in specialized neuromonitoring cages equipped for simultaneous performance of VNS, video-EEG monitoring and microdialysis sampling in freely moving conditions. Rats were connected to (i) a custom-built digital video-EEG monitoring system and (ii) an external current stimulator (NCP, model 100; Cyberonics Inc., Houston, TX, USA) via an electrical swivel (Plastics One, Roanoke, VA, USA). The microdialysis cannula obturator was replaced by a microdialysis probe (CMA12; 3 mm membrane length; theoretical cut-off 20 kDa; CMA/Microdialysis; Solna). The microdialysis probe was continuously perfused with modified Ringer's solution at a flow rate of 2 μL/min. A 15–20 h interval between probe implantation and the beginning of the experiment was respected to ensure integrity of the blood–brain barrier (Benveniste 1989), absence of excessive reactive gliosis in the tissue surrounding the microdialysis probe (Georgieva *et al.* 1993) and stable basal neurotransmitter dialysate concentrations (Clapp-Lilly *et al.* 1999).

Experimental design

In all animals, limbic seizures were evoked by intrahippocampal perfusion of the muscarinic agonist pilocarpine (10 mM) via the microdialysis probe. This rodent model for acute limbic seizures (Millan *et al.* 1993) has been extensively used in our laboratory (Smolders *et al.* 1997; Meurs *et al.* 2008), and is characterized by the sequential development of typical behavioural patterns, electrographic activity and neurochemical alterations (Meurs *et al.* 2008).

In a first series of experiments, we studied the effects of VNS on hippocampal neurotransmitter levels and pilocarpine-induced limbic seizure activity. Based on the results of these experiments, we conducted a second series of experiments in which we tested whether the effects of VNS on pilocarpine-induced seizure activity could be reversed by intrahippocampal application of a selective α_2-adrenoreceptor antagonist.

During each experiment, the microdialysis probe was continuously perfused at a flow-rate of 2 μL/min and hippocampal dialysate samples (40 μL) were collected every 20 min. Protocols for each experimental group are shown in Fig. 1 and detailed in the following section.

Effect of VNS on limbic seizure activity and hippocampal neurotransmitter levels

SHAM group (n = 7): the probe was perfused with modified Ringer's solution (R) during the first 15 collection periods. During collection periods 16 and 17, 10 mM pilocarpine was added to the perfusion fluid. Subsequently, perfusion fluid was switched back to modified Ringer's solution and samples were collected for another five collection periods (collection periods 18–22).

VNS group (n = 12): protocol as for the SHAM rats except but VNS was performed from the beginning of collection period 10 until the end of the experiment (i.e. collection period 22). VNS was delivered with the following stimulation parameters: frequency = 30 Hz; intensity = 1 mA; pulse width = 250 μs; duty cycle = 7 s ON–18 s OFF.

Effect of α_2-adrenoreceptors antagonism on the seizure-suppressing effect of VNS

SHAM-SKF group (n = 10): from collection period 7 until the end of the experiment, 1 nM SKF-86466 was added to the perfusion

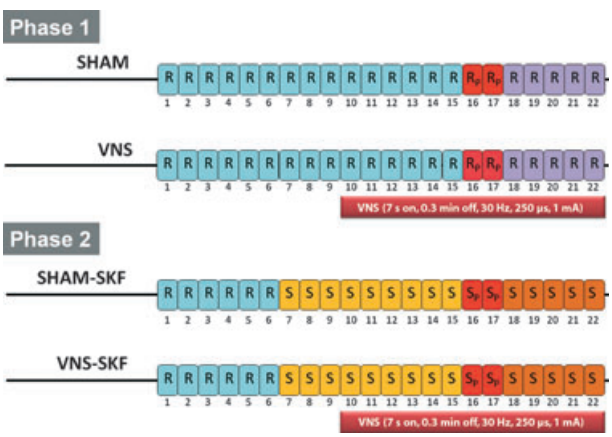


Fig. 1 Schematic representation of experimental protocols: Every square represents a 20-min perfusion of the microdialysis probe with: R = modified Ringer's solution; R_P = 10 mM pilocarpine dissolved in modified Ringer's solution; S = 1 nM SKF-86466 (α_2-receptor antagonist) dissolved in modified Ringer's solution and S_P = 10 mM pilocarpine and 1 nM SKF-86466 dissolved in modified Ringer's solution.

fluid. During collection period 16 and 17, perfusion was switched to a mixture of 10 mM pilocarpine and 1 nM SKF-86466. Subsequently, perfusion fluid was switched back to the 1 nM SKF-86466 solution and samples were collected for another five collection periods (collection periods 18–22). The SKF-86166 perfusate concentration was selected based on the published affinity constant (K_i = 6 nM) (Heal *et al.* 1995). This concentration was compensated for expected probe recovery (approximately 10%) and multiplied by 2 to obtain full receptor antagonism.

VNS-SKF group (n = 8): protocol as for the SHAM-SKF group, but VNS (frequency = 30 Hz; intensity = 1 mA; pulse width = 250 μs; duty cycle = 7 s ON–18 s OFF.) was delivered from collection period 10 onwards.

Video–EEG recording

All rats were monitored with continuous video-EEG recording throughout the experiment. Behavioural changes indicative of seizure activity were scored by reviewing video recordings. For each collection period, behavioural changes were rated on a seizure severity scale based on Racine's scale, which was adapted to include all behavioural changes observed in focal limbic seizure models: (0) normal, non-epileptic activity; (1) mouth and facial movements, hyperactivity, grooming, sniffing, scratching, wet dog shakes; (2) head nodding, staring, tremor; (3) forelimb clonus; forelimb extension; (4) rearing, salivating, tonic-clonic activity; (5) falling, status epilepticus. For each of the seven collection periods following the start of the pilocarpine administration (i.e. collection periods 16–22), the highest seizure severity score (SSS) was retained. Total SSS (TSSS) for each animal was calculated as the sum of the SSS, and used as a measure for seizure severity throughout the experiment. Hippocampal and cortical EEG recordings were reviewed to determine the latency to occurrence of the first epileptiform activity (spikes) and the total duration of the epileptiform activity after the start of the pilocarpine infusion (collection periods 16–22).

Microdialysate analysis

Dialysate samples were split for analysis of monoamines (dopamine, serotonin, noradrenaline) (25 μL) and GABA (15 μL). For monoamine analysis, we performed an off-line microbore liquid chromatography assay (C8, 5 μm; 100 × 1 mm) based on ion-pair reversed phase chromatography, coupled to single-channel electrochemical detection with a low oxidation potential (+450 mV vs. Ag/AgCl) (Decade, Antec, Leiden, the Netherlands), as has previously been described in detail (Smolders *et al.* 2008). For the analysis of GABA, we performed pre-column derivatization with *o*-phtalaldehyde/2-methyl-2-propanethiol and iodoacetamide followed by reversed-phase isocratic microbore liquid chromatography (C8, 5 μm; 100 × 1 mm; Unijet, Bioanalytical Systems, Warwickshire, UK) and amperometric detection, as has previously been described in detail (Smolders *et al.* 1995).

Data analysis

All statistical analyses were performed using SPSS 15 for Windows. Data are expressed as mean ± standard error of the mean. The significance level for demonstrating differences between groups was set at $\alpha = 0.05$. To evaluate VNS-induced changes in hippocampal neurotransmitter levels, a Student's t-test for paired comparison was used. In the VNS group, mean neurotransmitter levels during baseline collections 4–9 were compared with the mean neurotransmitter levels during the six collection periods after the start of VNS and prior to pilocarpine perfusion (collection periods 10–15). In the VNS-SKF group, mean neurotransmitter levels in the three collections during SKF infusion (collection periods 7–9) were compared with the mean neurotransmitter levels during the six collection periods in which VNS and SKF were co-administered prior to pilocarpine infusion (collection periods 10–15).

The effect of VNS on pilocarpine-induced seizure activity was determined by comparing SSS and TSSS (Mann–Whitney U-test), and latency to epileptiform discharges and duration of epileptiform discharges on the hippocampal EEG (Student's t-test for comparison of independent samples) between the SHAM and VNS group. Pearson correlation tests were performed to analyze correlation between VNS-induced increase of hippocampal NAD levels and seizure parameters (severity, duration and latency).

The effect of hippocampal α_2-adrenoreceptors antagonism on pilocarpine-induced seizures and on the seizure-suppresing effect of VNS were assessed by comparing TSSS (Kruskal–Wallis ANOVA followed by Mann–Whitney *U post hoc* tests using adjusted P-levels after Bonferroni correction), and latency and duration of epileptiform discharges on hippocampal EEG (one-way ANOVA followed by Bonferroni-Dunn *post hoc* tests for pairwise comparison) between the relevant groups.

Results

Effect of VNS on limbic seizure activity and hippocampal neurotransmitter levels

In the SHAM-treated group, perfusion of a 10 mM pilocarpine solution through the hippocampal microdialysis probe during 40 min elicited a host of behavioural changes including increased exploratory behaviour, wet dog shakes and excessive grooming, beginning 15–20 min after the start of pilocarpine administration. Approximately 30–60 min following pilocarpine, these changes progressively evolved into recurring bouts of staring, mouth and facial movements, forelimb clonus, rearing, salivation and rarely falling. Individual behavioral seizures typically lasted between 5 s and 1 min. EEG showed continuous epileptic spikes and intermittent rhythmic epileptiform discharges in the hippocampus, which occasionally generalized to the cortex (Fig. 2a). None of the animals developed a status epilepticus or died.

In the VNS-treated group, pilocarpine-induced behavioural changes indicative of seizure activity were significantly less severe than in the SHAM group (Table 1). Overall seizure severity was significantly lower in the VNS-treated group (TSSS = 5 ± 1) compared with the SHAM group (TSSS = 14 ± 2) ($p < 0.01$). The latency between the start of pilocarpine administration and the occurrence of epileptiform activity on the hippocampal EEG was significantly prolonged in VNS-treated rats (26 ± 4 min) compared with SHAM-treated rats (12 ± 4 min) ($p < 0.05$, Table 1). Moreover, the total duration of epileptiform activity on the hippocampal

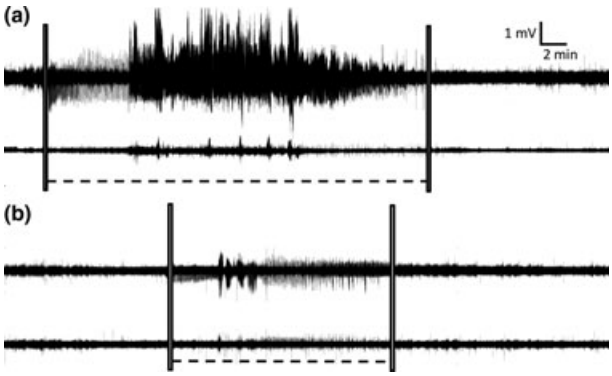


Fig. 2 Representative sample of EEG recorded from SHAM- (a) and VNS-treated rats (b). The upper traces represent EEG recorded from the left hippocampal depth electrode. The lower traces represent EEG recorded from an epidural electrode positioned over the right frontal cortex. EEG recordings are shown from the start of intrahippocampal pilocarpine infusion until the end of the experiment (i.e. collection periods 16–22, 140 min). The first vertical line represents the onset of epileptiform activity on hippocampal EEG. The second vertical line represents the end of epileptiform activity on hippocampal EEG. Both in SHAM- and VNS-treated rats, intrahippocampal infusion of pilocarpine-induced epileptic spikes and intermittent rhythmic epileptiform discharges, visible on hippocampal EEG and occasionally also on cortical EEG. In VNS-treated animals (b), the latency to first epileptiform activity was significantly increased and the total duration of epileptiform activity was significantly decreased compared with SHAM-treated animals (a).

EEG was significantly shorter in VNS-treated rats (67 ± 12 min) compared with SHAM-treated rats (111 ± 8 min) ($p < 0.05$, Table 1). A representative sample of the EEG recorded from hippocampus and cortex in SHAM- and VNS-treated rats is shown in Fig. 2.

During VNS, the mean dialysate concentration of noradrenaline increased by 69 ± 16% (0.122 ± 0.012 nM) compared with baseline (0.075 ± 0.008 nM) ($p < 0.01$). VNS had no significant effect on the dialysate concentration of serotonin, dopamine and GABA (Fig. 3).

VNS-induced increases in the dialysate concentration of noradrenaline were positively correlated with the latency to epileptiform activity on hippocampal EEG ($R^2 = 0.82$; $p < 0.01$), and negatively correlated with the total duration of epileptiform activity on hippocampal EEG ($R^2 = 0.62$; $p < 0.01$) and TSSS ($R^2 = 0.81$; $p < 0.01$) (Fig. 4a–c).

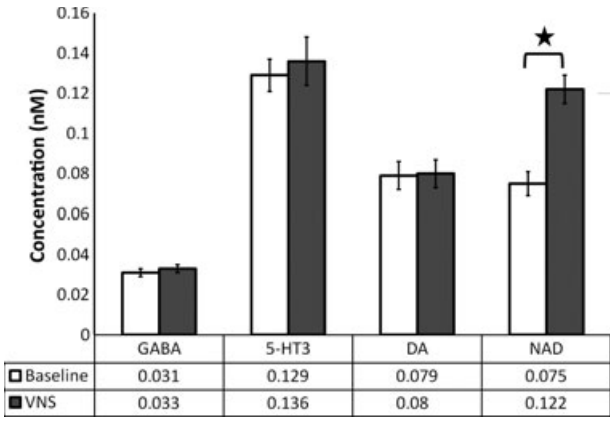
Animals could be divided into two distinct groups on the basis of the noradrenergic and anticonvulsive effects of VNS. Rats with a VNS-induced increase in hippocampal noradrenaline concentration of at least 70% (seven of 12 rats, subsequently referred to as 'responders') had a pronounced reduction in seizure severity. Conversely, animals with a VNS-induced increase in hippocampal noradrenaline concentration of less than 70% (five of 12 rats, subsequently referred to as 'non-responders') were not protected from pilocarpine-induced seizures. No significant correlation was found between baseline hippocampal NAD levels and seizure parameters (severity, latency and duration).

Effect of α_2-adrenoreceptor antagonism on the seizure suppressing effect of VNS

Intrahippocampal infusion of 1 nM SKF-86466 alone induced an increase in mean noradrenaline dialysate concentration of 97 ± 14% in the VNS-SKF group and 84 ± 28% in the SKF group (Table 1). VNS-induced increases in noradrenaline in the VNS-SKF group were determined by comparing mean noradrenaline concentration in the three collections during SKF infusion (collection periods 7–9) to the mean noradrenaline concentration during the six dialysate collections in which VNS and SKF were co-administered prior to pilocarpine infusion (collection periods 10–15). VNS produced an additional increase in mean noradrenaline dialysate concentration of 57 ± 12% compared noradrenaline contrations during administration of SKF alone. Of a total of eight rats, four had a VNS-induced

Table 1 Overview of seizure parameters (severity, latency and duration) in all experimental groups. In VNS and VNS-SKF group, a subdivision was made between non-responders and responders based on VNS-induced noradrenaline increase. In the VNS-SKF group, noradrenaline levels prior to VNS were already increased compared with baseline because of intrahippocampal infusion of 1 nM SKF-86466

Treatment group	Seizure severity (TSSS)	Seizure latency (min)	Seizure duration (min)	SKF-induced NAD increase (%)	VNS-induced NAD increase (%)
SHAM	14 ± 2	12 ± 4	111 ± 8	N.A.	N.A.
VNS	5 ± 1	26 ± 4	67 ± 12	N.A.	69 ± 16
Non-Resp.	10 ± 1	10 ± 1	106 ± 10	N.A.	11 ± 9
Resp.	2 ± 1	38 ± 3	40 ± 10	N.A.	110 ± 9
VNS-SKF	11 ± 1	20 ± 5	100 ± 8	97 ± 14	57 ± 12
Non-Resp.	11 ± 3	9 ± 3	103 ± 27	101 ± 18	31 ± 11
Resp.	12 ± 1	24 ± 6	99 ± 7	84 ± 14	83 ± 7
SHAM-SKF	13 ± 2	20 ± 5	95 ± 2	84 ± 28	N.A.



	GABA	5-HT3	DA	NAD
Baseline	0.031	0.129	0.079	0.075
VNS	0.033	0.136	0.08	0.122

Fig. 3 Effect of VNS on hippocampal levels of GABA, serotonin (5HT), dopamine (DA) and noradrenaline (NAD). In the VNS group, mean neurotransmitter levels during baseline collections 4–9 were compared with the mean neurotransmitter levels during the six collection periods after the start of VNS and prior to pilocarpine perfusion (collection periods 10–15). Average dialysate concentrations are presented in the table under the graph (in nM). No significant changes could be demonstrated for GABA, 5HT and DA. VNS did induce a significant increase of hippcampal NAD levels. *Statistical significant difference ($p < 0.05$).

increase in hippocampal noradrenaline of ≥ 70% (further defined as responders).

Because the aim of these experiments was to determine the involvement of increased hippocampal noradrenaline in the seizure-suppressing effect of VNS, only responders were included in the statistical analysis of seizure activity.

In responders, intrahippocampal administration of the selective α_2-adrenoreceptor antagonist SKF-86466 completely reversed the seizure-suppressing effect of VNS. Latency to epileptiform discharges, total duration of epileptiform activity and TSSS were similar for VNS-SKF- and SHAM-SKF-treated rats (Fig. 5). Seizure duration and severity in the VNS-SKF group were significantly increased compared with VNS-treated rats, and were comparable to what was observed in SHAM-treated rats (Fig. 5b and c).

Discussion

The main findings of this study are (i) that VNS induces an increase in the extracellular hippocampal concentration of noradrenaline, but not of dopamine, serotonin and GABA; (ii) that VNS prevents the development of pilocarpine-induced limbic seizures only in those rats in which hippocampal noradrenaline increased by at least 70%; and (iii) that selective α_2-adrenoreceptor antagonism in proximity of the seizure focus abolishes the seizure-suppressing effect of VNS. Taken together, these findings provide convincing evidence for the existence of a strong causal link between the seizure-suppressing effect of VNS and increased hippocampal noradrenergic signaling.

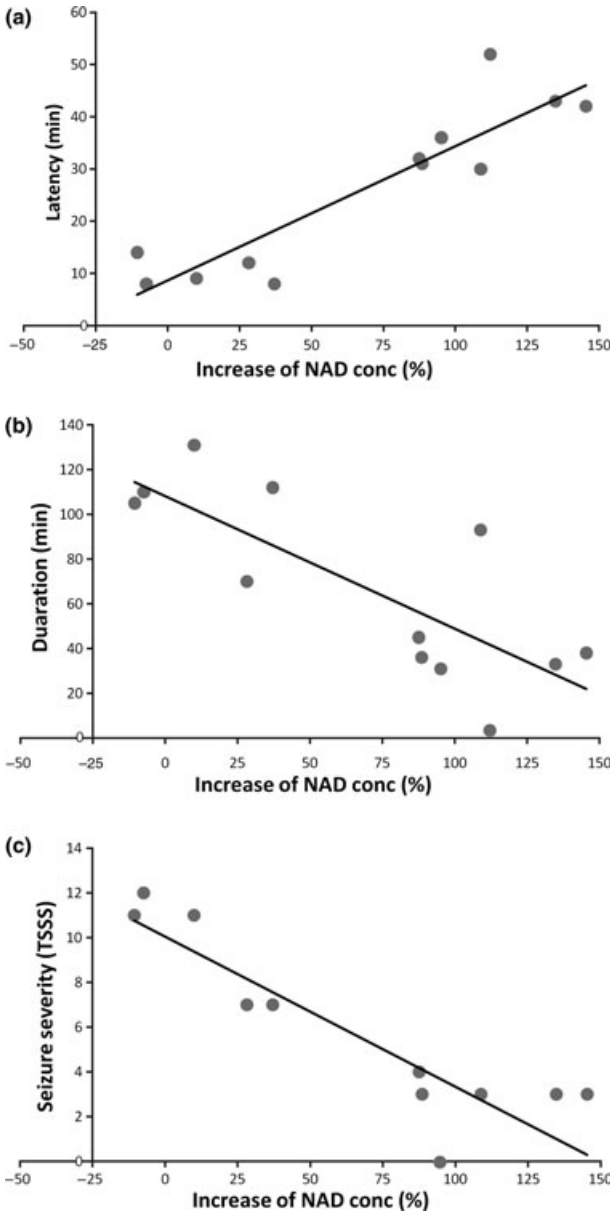


Fig. 4 Correlation between VNS-induced changes in hippocampal noradrenaline levels and seizure-suppressing effects. (a) VNS-induced increases in hippocampal noradrenaline levels were positively correlated with the latency to epileptiform activity on hippocampal EEG after starting the pilocarpine infusion. VNS-induced increases in hippocampal noradrenaline levels were negatively correlated with (b) the total duration of epileptiform activity on hippocampal EEG and (c) clinical seizure severity represented by TSSS.

Roosevelt *et al.* (2006) have previously reported a bilateral increase in noradrenaline levels in the cortex (39%) and the hippocampus (28%) in response to 1 h of VNS (20 Hz, 1 mA, 500 μ, 30 s ON–10 min OFF). In our hands, VNS produced a more than twofold higher increase (69%) in extracellular hippocampal noradrenaline. This may be due to

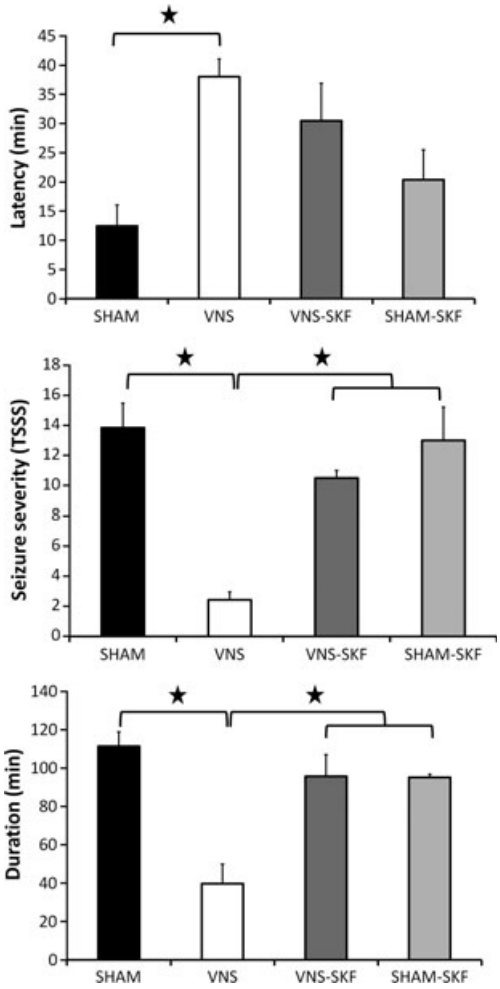


Fig. 5 Effect of α_2-adrenoreceptor antagonism on the seizure-suppressive effects of VNS. Intahippocampal infusion of the selective α_2-adrenoreceptor antagonist SKF-86466 completely blocked VNS-induced effects on latency to epileptiform discharges (a), total duration of epileptiform activity (b) and clinical seizure severity represented by TSSS (c) because these outcome parameters were similar for VNS-SKF and SHAM-SKF rats. *Statistical significant difference ($p < 0.05$).

the fact that we used a more intensive stimulation protocol (30 Hz, 1 mA, 250 μs, 7 s ON–18 s OFF), resulting in the delivery of a total of 30 240 electrical pulses to the vagus nerve compared with 3600 pulses in the study by Roosevelt and colleagues. The duty cycle used in our experiments (7 s ON–18 s OFF) is referred to as 'rapid cycling', and is used to treat patients in whom VNS with a duty cycle of 30 s ON–10 min OFF did not sufficiently improve seizure control (Liporace *et al.* 2001; Labar 2004).

In our hands, VNS suppressed pilocarpine-induced limbic seizures most strongly in those rats with the largest increase in hippocampal noradrenaline. Conversely, rats in which VNS did not increase hippocampal noradrenaline exhibited the most severe seizures. A noradrenaline increase of at least 70% seems to be associated with suppression of limbic seizures. Interestingly, in previous work, we found that hippocampal extracellular noradrenaline increased by 70% in rats that were treated with the selective noradrenaline reuptake inhibitor, maprotiline, at the minimal dose required to suppress pilocarpine-induced seizures (Clinckers *et al.* 2010).

Based on these observations, we hypothesized that VNS prevents the development of limbic seizures by increasing hippocampal noradrenaline. To test this hypothesis, we verified whether blockade of adrenoreceptors in proximity of the seizure focus would reverse the anticonvulsive effect of VNS. We chose to use a selective α_2-adrenoreceptor antagonist in these experiments, based on previous work in which we showed that application of this antagonist was sufficient to completely reverse the anticonvulsive effect of increased hippocampal noradrenaline (Clinckers *et al.* 2010).

We found that in rats with a VNS-induced increase in hippocampal noradrenaline of ≥ 70%, concomitant intrahippocampal administration of the selective α_2-adrenoreceptor antagonist SKF-86466 abolished the anticonvulsive action of VNS. This finding strongly supports the hypothesis that the seizure suppressing effect of VNS is at least partly mediated by increased hippocampal noradrenaline concentration and increased hippocampal α_2-adrenoreceptor activation.

In our hands, only a subset of animals (seven of 12) exhibited an VNS-induced increase in hippocampal noradrenaline that was sufficient to produce an anticonvulsant effect. This 'responder-rate' is remarkably similar to what has been observed in clinical trials with VNS.

One small open-label study in patients with TLE and seizures originating independently from the left and right temporal lobes showed that VNS reduced seizure frequency by at least 50% in six of 10 patients (Alsaadi *et al.* 2001). Larger clinical trials that have evaluated the efficacy of VNS in various types of refractory epilepsy have consistently shown that VNS is ineffective at reducing seizures in approximately one-third of patients (Ben-Menachem *et al.* 1994; Handforth *et al.* 1998; Vonck *et al.* 2004; De Herdt *et al.* 2007).

Several, although not necessarily similar, factors could account for the inter-subject variability of the response to VNS in our experiments and in patients suffering from TLE. First, there could be a difference in vagus nerve activation in response to VNS. Although an electrode lead break and stimulator defect was excluded by measuring impedance, it is possible that VNS does not activate vagus nerve fibers because of injury of the nerve induced by induced electrode implantation. Temporary nerve damage could explain the delayed effect of VNS in some patients with epilepsy implanted with a VNS system. Second, genetic variability and differences in external and/or internal environment could lead to differences in the number of noradrenergic neurons, their afferent and/or efferent projections and synaptic strengths within these noradrenergic neuronal networks. This intrinsic variation in the noradrenergic neural network could

underlie the variable release of noradrenaline in response to VNS. One argument against this second hypothesis is that this study shows that differences in baseline noradrenaline levels do not explain the variation in clinical response to VNS indicating that baseline hippocampal NAD level is no critical indicator of VNS-mediated anti-convulsant effect.

Given the causal relationship between increased hippocampal noradrenaline and suppression of limbic seizures in response to VNS in rats, VNS-induced increases in hippocampal noradrenaline may be a useful biomarker for the efficacy of VNS in human TLE. If used in combination with a non-invasive technique to deliver VNS [f.i. transcutaneous activation of the vagus nerve (Dietrich *et al.* 2008)], a biomarker for the efficacy of VNS could help clinicians to reliably identify responders prior to surgical implantation of a VNS device, and to determine optimal stimulation parameters in a rational way.

Conclusion

The main findings of this study are (i) that VNS induces an increase in the extracellular hippocampal concentration of noradrenaline, but not of dopamine, serotonin and GABA; (ii) that VNS prevents the development of pilocarpine-induced limbic seizures only in those rats with VNS-induced increases in hippocampal noradrenaline of at least 70%; and (iii) that selective α_2-adrenoreceptor antagonism in proximity of the seizure focus abolishes the seizure-suppressing effect of VNS. Taken together, these findings provide convincing evidence for the existence of a strong causal link between increased noradrenergic signaling and the anticonvulsant effect of VNS.

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