



Original Research

Acute Vagal Nerve Stimulation Lowers $\alpha 2$ Adrenoceptor Availability: Possible Mechanism of Therapeutic Action

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ABSTRACT

Background: Vagal nerve stimulation (VNS) emerged as an anti-epileptic therapy, and more recently as a potential antidepressant intervention.

Objective/hypothesis: We hypothesized that salutary effects of VNS are mediated, at least in part, by augmentation of the inhibitory effects of cortical monoaminergic neurotransmission at appropriate receptors, specifically adrenoceptors. Our objective was to measure the effect of acute VNS on $\alpha 2$ adrenoceptor binding.

Methods: Using positron emission tomography (PET), we measured changes in noradrenaline receptor binding associated with acute VNS stimulation in six anesthetized Göttingen minipigs. We used the selective $\alpha 2$ adrenoceptor antagonist [¹¹C]yohimbine, previously shown to be sensitive to competition from the receptor's endogenous ligands, as a surrogate marker of monoamine release. PET records were acquired 4–6 weeks after the implant of a VNS electrode in minipigs before and within 30 min of the initiation of 1 mA stimulation. Kinetic analysis with the Logan graphical linearization generated tracer volumes of distribution for each condition. We used an averaged value of the distribution volume of non-displaceable ligand (V_{ND}), to calculate binding potentials for selected brain regions of each animal.

Results: VNS treatment markedly reduced the binding potential of yohimbine in limbic, thalamic and cortical brain regions, in inverse correlation with the baseline binding potential.

Conclusion: The result is consistent with release of noradrenaline by antidepressant therapy, implying a possible explanation for the antidepressant effect of VNS.

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Introduction

Vagal Nerve Stimulation (VNS) is a form of stimulation of the brain which has anticonvulsant effects in animal models of epilepsy [1,2] and in humans with seizures [3]. As such, it has been approved as an adjunct treatment for partial complex seizures since 1994 in Europe and since 1997 in the USA.

Follow-up of VNS-treated epileptic patients revealed improvements in mood in the majority of patients, including patients with no apparent changes of seizure frequency or intensity. These observations prompted further studies of the possible antidepressant effect of VNS. Since then, VNS has been shown to exert some antidepressant effects in a model of depression in rats and in

depressed humans [4–7]. As a result, the Food and Drug Administration approved VNS in 2005 for adjunctive therapy in treatment-resistant depression. The exact therapeutic mechanisms of VNS in epilepsy and mood disorders are unknown. However, evidence from the abundant literature on specific antidepressant and anti-epileptic therapies, and from the known anatomical connections of the vagus nerve in brainstem and pons, makes it likely that effects of VNS are mediated by monoaminergic neurotransmission. Through the nucleus tractus solitarius, the ascending pathways from the vagus nerve connect indirectly to the locus coeruleus (LC), the main origin of noradrenergic innervation of the cortex [8] and the hippocampus [9]. While it is unknown whether the vagal influences on the LC are excitatory or inhibitory, lesions of the LC confirm a role of this nucleus in seizures [10]. Observations of effects of pharmacological antidepressant therapy suggest that both serotonergic and noradrenergic (NA) neurotransmissions are affected in mood disorders. It is possible that modulation of noradrenergic neurotransmission plays a role in at least some of the therapeutic effects of VNS.

We recently developed [¹¹C]yohimbine, an antagonist primarily of the α_2 adrenoreceptors, as a tracer of noradrenergic neurotransmission by means of PET, and validated its use in pigs [11]. In pharmacological doses, yohimbine is not exclusively selective for α_2 receptors, as it has been reported to bind with moderate or weak affinity to other receptors *in vitro*, such as D₂, α_1 and 5HT_{1A}, albeit with 5–10-fold lower affinity [12–14]. However, in tracer concentrations, yohimbine is highly selective for α_2 sites *in vivo* [11]. Central α_2 NA receptors are expressed presynaptically on noradrenergic neurons in the LC, where they control the release of the neurotransmitter, and postsynaptically in the widely distributed projection areas of the NA neurons throughout the cortex where they modulate signaling pathways by limiting signal dissipation in dendritic spines [15]. At rest, the distribution of labeled yohimbine in the living pig brain is consistent with the known *in vitro* distribution of α_2 receptors, with density in cortex and thalamus greater than density in mesencephalon, which in turn has greater density than cerebellum, pons, and medulla [16]. Furthermore, we recently demonstrated displacement of yohimbine binding in response to amphetamine challenge in pigs and rats, suggesting competition with the endogenous ligand [17,18]. These observations, together with reports that stimulation of the vagal nerve in rodents induces NA release [19], prompted the current test *in vivo* of the hypothesis that the acute effects of a human VNS device are consistent with the release of NA or other monoamines, or both, in a healthy brain.

Göttingen minipigs are large animals in which the device designed for use in humans easily is implanted, and stimulation is executed with similar parameters, facilitating the translation of these observations for clinical application. To test the hypothesis, we implanted VNS electrodes on the left vagus nerve of minipigs and imaged the brain with [¹¹C]yohimbine before and shortly after stimulation to allow us to determine the effect of VNS on the binding of the tracer.

Material and methods

Animals

Six 14-month old normal female Göttingen minipigs weighing 25–32 kg (Ellegaard Minipigs ApS, Dalmoose, Denmark) were used in the VNS study (labeled VNS1–VNS6), in accordance with a protocol approved by the Danish Animal Experimentation Inspectorate. The brain size (about 80 g) is adequate for imaging, and the existence of an MRI-based atlas allows co-registration of PET data for accurate analysis of regional distribution of the tracer. The minipigs were fed a restricted pellet diet (DIA plus FI, DLG, Aarhus, Denmark). They were

fasted overnight, with free access to tap water, prior to the VNS surgery and the PET scans. Environmental conditions in the animal facility were 20 °C and 50–55% relative humidity, and the air was changed 8 times every hour. Pigs were single housed in a 4.6 m² enclosure with fence-line contact with congeners.

VNS surgery and stimulation

The minipigs were pre-medicated with a mixture of 1.25 mg/kg midazolam and 6.25 mg/kg s-ketamine intramuscularly (IM). After placing of an ear vein catheter (21G Venflon), anesthesia was induced with a mixture of 1.25 mg/kg midazolam and 3.13 mg/kg s-ketamine intravenously (IV). The minipigs were intubated, and anesthesia was maintained on 3.7 mg/kg/h to 4.0 mg/kg/h propofol IV. The minipigs were mechanically ventilated with approximately 8 ml/kg/min of a mixture containing 1 O₂ and 2.2 medical air. Pulse, arterial oxygen (SaO₂) and body temperature were monitored during the whole procedure and a saline drip prevented dehydration. Analgesics such as Flunixin and Temgesic were administered for up to 5 days post-surgery to minimize pain and discomfort. The antibiotic Penovet was administered at least 30 min before the start of the procedure and then daily for up to 5 days post-surgery. The animal was placed supine and the head was immobilized and taped towards the right to expose the left neck area. The implant of the vagal nerve stimulator (Cyberonics Inc) was performed by an experienced neurosurgeon (SD) under sterile conditions using a modified human protocol. As in human subjects, the implant was placed on the left vagus in order to avoid the cardiac branches of the vagal nerve. The nerve was carefully dissected and exposed and the stimulating coils were wrapped gently around it. A second incision was placed on the left side of the back of the neck to create a pocket in which to slide the stimulating device. During the surgery, we tested the stimulator efficacy and connection with the control device by turning on the VNS for a 1-min period at 1 mA. The device was turned off again and remained off until the time of the PET studies. None of the animals developed side effects or infection from the surgical procedure.

The PET studies were performed 4–6 weeks after stimulator implant to allow the animal ample time for recovery from surgery and to ensure the lack of infection and adverse reactions. Baseline and experimental tomography occurred on the same day. This design ensured that the possible changes in the VNS ON condition could not be attributed to possible damage, trauma or inflammation of the vagus nerve due to surgery compared to baseline. It also reduced the variability due to positioning in the scanner and other physiological differences. The baseline study was performed first (baseline OFF condition) and the challenge study approximately 30 min after turning the stimulator on (ACUTE ON). The choice of stimulation intensity was based on the data by Roosevelt [19] reporting a significant increase in NA concentrations in both the cortex and hippocampus of rats at 1 mA stimulation intensity of the vagus nerve. Furthermore, this intensity is used during the surgical procedure for the testing of the stimulator and leads.

Tomography

The anesthesia regimen and preparation of the pigs for scanning was described in detail elsewhere [20] and was similar to the one described above for the VNS implant. The minipigs were positioned in a state of the art High-Resolution Research Tomograph (HRRT, CTI/Siemens) in a dorsal recumbent position, with the head immobilized with a custom device, and covered with a heating blanket set to maintain a stable body temperature. The HRRT permits the acquisition of 207 slices, 1.2 mm apart center to center and has a reconstructed resolution of about 2 mm FWHM. After a brief

transmission scan, the baseline scan (VNS OFF) was acquired. At the end of the acquisition, and about 25–30 min before the next dose of yohimbine, the VNS stimulator was turned ON at 1 mA. A second yohimbine scan was acquired with the same parameters. Table 1 summarizes the injection parameters for both conditions. The tracer was adjusted to a fixed volume of 10 ml with sterile saline and injected IV over 1 min. The catheter was then flushed with 10 ml sterile saline. The tomography started at injection and lasted 90 min in list mode. The two PET sessions were conducted at least 2 h apart (6 half-lives) so as to avoid the presence of residual tracer during the second scan. Appropriate correction factors were applied to the sinograms and the data were reconstructed in 17 frames of increasing duration from 1 min to 10 min using a Point Spread Function reconstruction algorithm (10 iterations).

Data analysis

The PET images were summed over the duration of the study and the summed image was co-registered to an average T1 weighted MRI atlas constructed from 22 minipig brains [21] using Montreal Neurological Institute software. Regions of interest included cerebellum, thalamus, striatum (caudate and putamen), cortical regions (temporal, frontal and occipital) and limbic regions (hippocampus and amygdala), all identifiable on the MRI of the minipig brain. The Logan graphical analysis [22] used the slope between 25 and 90 min to obtain an estimate of the total volume of distribution of the ligand (V_T).

As this study was part of a larger study protocol, in order to reduce the burden on the animals and the risk of infection, no arterial line was placed and the input function used in the data analysis was made from the average standard blood curve obtained from the arterial plasma data from 18 pigs, corrected for injected dose and weight. We previously validated this method, and the original studies showed that pigs do not possess the liver enzymes necessary to metabolize yohimbine [11]. Multiple studies with various ligands have demonstrated that the major source of variability in PET studies stems from the use of metabolite-corrected input functions [23–25]. Unfortunately, the widespread distribution of α_2 adrenoceptors throughout the entire brain precludes the use of an adequate non-specific tissue input function. We previously investigated the use of a white matter region but the small size of the swine brain does not permit to identify a white matter region of sufficient size that it is not extensively contaminated by partial voluming from adjacent gray matter regions. The use of a scaled standard plasma activity curve as input is further justified in pigs as they do not metabolize yohimbine.

In order to obtain a specific binding potential BP_{ND} , defined as $(V_T/V_{ND}) - 1$ according to accepted nomenclature [26], we used the average of the volume of distribution of the free plus non-specifically bound tracer V_{ND} measured in previous pharmacological displacement studies in 15 pigs and minipigs. The V_{ND} values were extracted from each animal with the Inhibition Plot [27] where inhibition V_T is plotted against baseline V_T . The average of these estimates of V_{ND} was 1.9 ml/cm³ (range: 0.7–2.38 ml/cm³) and is referred to below as V_{ND-A} . The BP_{ND} calculated with this average V_{ND} are shown as $BP_{ND-A} = (V_T/V_{ND-A}) - 1$.

Table 1

Average values \pm standard deviation for the specific activity (SA), the mass of injected yohimbine and the dose of injected tracer for the baseline and acute VNS scans.

	SA (GBq/ μ mol)	Mass (μ g)	Dose (MBq)
Baseline	105 \pm 66	1.63 \pm 1.12	332 \pm 49
Acute VNS	109.5 \pm 56	1.28 \pm 0.75	305 \pm 34

The V_T and BP_{ND-A} for the 6 pigs were analyzed using a two-way ANOVA with REGION and TREATMENT (VNS) as the factors, followed by a Bonferroni correction. Regional differences were measured using paired *t*-tests.

Results

Overall, acute VNS treatment markedly lowered yohimbine V_T and BP_{ND} (Fig. 1) throughout the brain. For both measures of yohimbine binding, the two-way ANOVA revealed no significant effect of REGION (Df: 8; $F = 1.85$, $P = 0.09$), but a very significant effect of TREATMENT (Df: 1, $F = 35.21$, $P < 0.0001$) without interaction (Df: 8; $F = 0.25$, $P = 0.98$) (Table 2). In this sample of 6 animals with 9 regions each, significance testing between region using Bonferroni correction for multiple comparisons only revealed a significant difference after acute VNS in the hippocampus ($P < 0.05$). However, a less stringent two-tailed paired *t*-test to determine significant VNS-induced changes for each region of the 6 pigs, revealed a significant difference in the thalamus ($P = 0.013$) and hippocampus ($P = 0.019$), with trends towards significant differences in the temporal, frontal, and occipital cortices ($P = 0.089$, 0.093, and 0.076, respectively) (Table 3).

The individual data revealed there was no change over test-retest reproducibility in the receptor availability in two animals (VNS3 and VNS4) with the lowest baseline values of V_T and BP_{ND-A} (Fig. 2). In contrast, there was a pronounced decline in the two measures of yohimbine binding, V_T and BP_{ND-A} , in the four remaining pigs (Tables 2 and 3) which is much greater than the test-retest reproducibility values for this tracer. For yohimbine, the averaged regional test-retest V_T data in 4 normal animals yielded an overall value of 5.8% across the entire brain, with a range of -5 to $+18\%$. The regional data had limited variability within subjects and ranged from $+3$ to $+7\%$ overall. These data suggest that the effect on tracer binding is a result of the VNS stimulation, and that the estimates of V_T during the dual injection of [¹¹C]yohimbine were stable for the duration of anesthesia.

Discussion

The significant decrease of yohimbine binding is consistent with results of rat brain microdialysis according to which stimulation of the vagus nerve rapidly raised synaptic NA concentration [19]. Furthermore, electrophysiology of rat brain undergoing VNS stimulation revealed a rapid increase of basal firing rates of neurons in the LC [28,29] and an increase in the percentage of neurons firing in bursts, even after 90 days of stimulation [30]. The PET results are in accord with previous microdialysis and challenge studies from our group, suggesting that yohimbine is sensitive to displacement by the receptor's endogenous ligands [17,18,31].

In the treatment of human epilepsy, VNS therapeutic protocols begin with a current of 0.25 mA, which progressively rises by 0.25 mA increments over a period of several weeks to a final current of 2 mA. The duty cycle parameter regulates the duration of the stimulation ON and OFF periods, and is often 30 s ON and 5 min OFF in human treatment. During the present acute scan, the stimulation ON period was 21 s and the OFF period was 0.8 min at a current of 1.0 mA, which is higher than the recommended starting current in human treatments, but similar to the current used to test the device during surgical implantation in human subjects. Aside from the use of the same acute clinical protocol, the present current intensity was chosen because the rat microdialysis revealed consistent increases of extracellular NA in cortex and hippocampus in response to VNS at 1.0 mA, but less consistent responses at lower stimulation levels [19].

Interestingly, the results of this sample of 6 animals suggest that VNS exerts a significant inhibitory influence mostly in limbic,

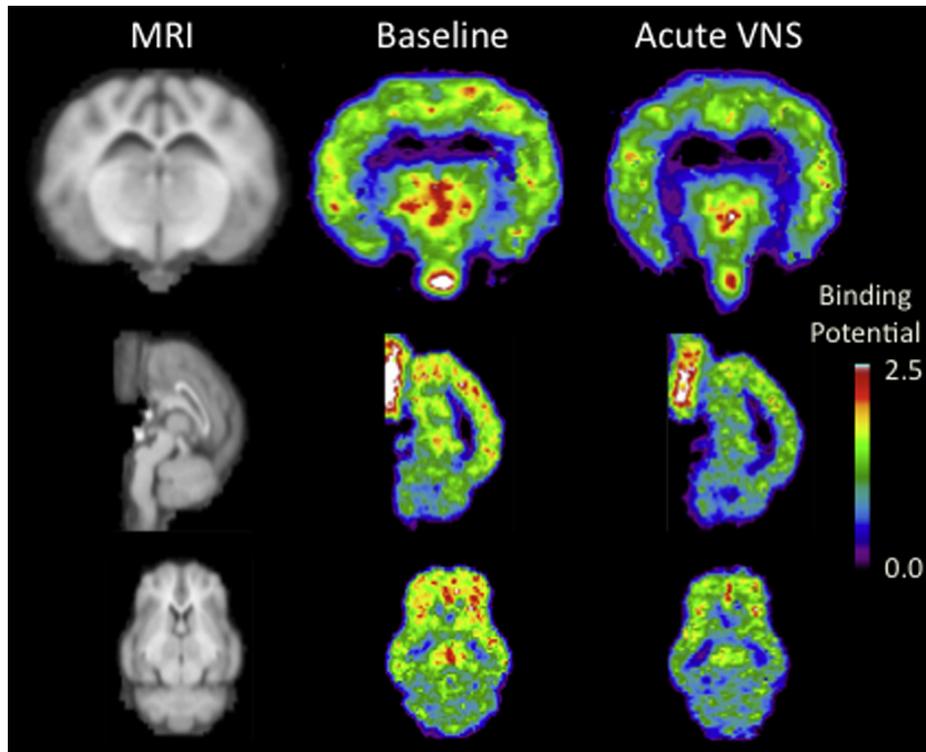


Figure 1. Acute vagal nerve stimulation displaces [^{11}C]yohimbine binding to the α_2 adrenoceptors in minipig brain. Parametric maps of the average binding potential of the 6 pigs at baseline (middle) and after 30 min of acute stimulation (right) are shown. The left column shows the corresponding MRI images.

thalamic and cortical regions. The central distributions of monoamines are well documented with a prevalence of DA terminals in striatum while NA terminals predominate in cortical, limbic and thalamic regions. Despite the low density of DA innervation in non-striatal areas, it is however possible that part of the effect of VNS on yohimbine binding is mediated by release of DA as well as of NA. Indeed, electrical stimulation of the LC elicits both NA and DA release from NA terminals in rat cortex [32,33] and many studies support crosstalk between the two ligands and their respective receptors [12]. The relative affinities of DA and NA for α_2 receptors in pigs are unknown. It is also unknown whether the interaction of DA with the α_2 receptors is regionally specific. It is however of note that while striatal NA innervation is sparse, the striatum (and cerebellum) display measurable yohimbine binding. Both contain an abundance of α_2 receptors, mostly of the C subtype (60–70%) [34,35] and some studies also suggest that the preferred ligand of the $\alpha_2\text{C}$ receptors is DA [36]. The apparent discrepancy between the distribution of NA terminals and the relatively large but non significant effect of VNS stimulation in striatal regions may be due to a combination of the lower NA innervation and preference of the $\alpha_2\text{C}$ receptors for DA.

The inhibitory effect of VNS on yohimbine binding in limbic and cortical regions of the minipig brain is consistent with the well-known role of NA in therapy of epilepsy and depression, two

indications for which VNS is an approved therapeutic option. NA is thought to reduce neuronal excitability in epilepsy and hence to be protective against the onset of seizures, as NA projections from the LC provide a tonic inhibitory action on the kindling of epilepsy. Increased levels of NA significantly attenuate seizure kindling, whereas damage to the LC facilitates kindling [37,38]. Monoamine depletion by 6-hydroxydopamine also promotes kindling [39], which can be suppressed by NA-rich cell suspensions [40]. Changes in the levels of NA and other monoamines in limbic brain structures relevant to kindling have been observed [41], and elevated levels of NA and other monoamines have anticonvulsant effects [42,43]. Lower baseline concentrations of monoamines in the LC and amygdala are correlated with increased occurrence of seizures and increased duration of after-discharge in cats.

The insignificant decline of $\text{BP}_{\text{ND-A}}$ in amygdala following VNS was thus surprising. However, this region is small and difficult to identify accurately and reliably in pigs. Due to size and location, partial volume effects in the limbic regions probably diluted the signal from the tracer in this small group of animals, especially taking into consideration that we did not have individual MR images of each animal and therefore used the population atlas of the minipig brain for co-registration.

Evidence that lesions of the LC block the anticonvulsant effects of VNS in rats [10] suggests that the anti-epileptic effects of VNS

Table 2
Total volume of distribution (V_T) average of all 9 regions (standard deviation) for the 6 animals (VNS1–VNS6) and the percentage change from baseline.

	VNS1	VNS2	VNS3	VNS4	VNS5	VNS6
$V_{T-\text{Base}}$ (mL/cm 3)	5.87 (0.78)	6.09 (0.64)	3.91 (0.47)	3.64 (0.36)	4.88 (0.59)	4.30 (0.62)
$V_{T-\text{VNS}}$ (mL/cm 3)	5.13 (0.75)	4.81 (0.53)	3.84 (0.62)	3.78 (0.46)	4.08 (0.43)	3.75 (0.41)
% change	–15	–21	–2	3	–16	–14

Table 3

Average binding potential and standard deviation (Std) calculated using previously determined V_{ND-A} (1.9 ml/cm³) in each pig (VNS1–VNS6) for each of the 9 regions considered.

Brain region	Baseline		VNS		P
	Average	Std	Average	Std	
Temporal cortex	1.726264	0.636525	1.429208	0.358957	0.089*
Frontal cortex	1.883539	0.658552	1.549222	0.415241	0.093*
Occipital cortex	1.431518	0.572734	1.172405	0.33973	0.076*
Amygdala	1.352269	0.253913	1.144229	0.264166	0.310
Hippocampus	1.323871	0.563089	0.89879	0.356277	0.019**
Thalamus	1.829433	0.533953	1.497007	0.485067	0.013**
Putamen	1.613716	0.647435	1.371626	0.328408	0.231
Caudate nucleus	1.281794	0.612115	0.976089	0.249771	0.135
Cerebellum	1.20597	0.541147	1.016288	0.317829	0.158

The P value from the paired *t*-tests comparing baseline and VNS data is indicated in the last column (P).** indicates significant difference and * indicates a trend.

requires an intact noradrenergic neurotransmission. Thus, with the evidence of an effect of VNS on NA or other monoamine release estimated here over a period of time, we hypothesize that the therapeutic effects of VNS could be explained by maintenance of levels of NA (and DA). This conclusion is based on the apparent lack of effect in two animals in which the baseline volumes of distribution and binding potentials were already low (Table 2). One physiological explanation is that the NA system of these animals was not further responsive to the stimulation of the vagus nerve potentially due to a high baseline level of noradrenergic activity at α_2 adrenergic receptors (Fig. 2). Alternatively, the absence of effect could be due in part to a number of technical issues, including damaged leads, failure of the stimulator or the battery or poor connections. At the end of the study, the stimulator itself was tested and was found to be working properly in all of the animals. It was not possible to dissect out the leads and verify their positions due to significant tissue growth in the neck area. Thus, lead failure cannot be excluded. Technical failures, however, cannot explain the low baseline values in the two unresponsive animals.

The PET data is also relevant to the role of NA in depression. In major depression, the LC reveals increased density of α_2 adrenergic receptors and decreased density of NA transporters [44,45], suggesting fewer terminals and absent release of NA in the target

regions of depressed patients. Major depression is accompanied by increased receptor numbers in the hippocampus and cerebral cortex [46], and increased α_2 adrenergic receptor binding sites are found in the LC and limbic and cortical brain regions of suicide victims [46–50] where α_2 adrenoreceptor mRNA levels are also higher by 43% in the prefrontal cortex [51]. Antidepressant drugs and electroconvulsive therapy lower binding of [³H]idazoxan and [³H]RX821002 to α_2 adrenergic receptors *in vitro* [52,53] and of [¹¹C]yohimbine *in vivo* [54], consistent with the findings of the present VNS-stimulated minipigs.

In conclusion, acute VNS decreased α_2 adrenoceptor binding in anesthetized minipigs, suggesting increased noradrenaline release in response to VNS. The result is consistent with previous studies demonstrating release of noradrenaline by pharmacological and brain stimulation antidepressant therapies, implying a possible explanation for the antidepressant effect of VNS.

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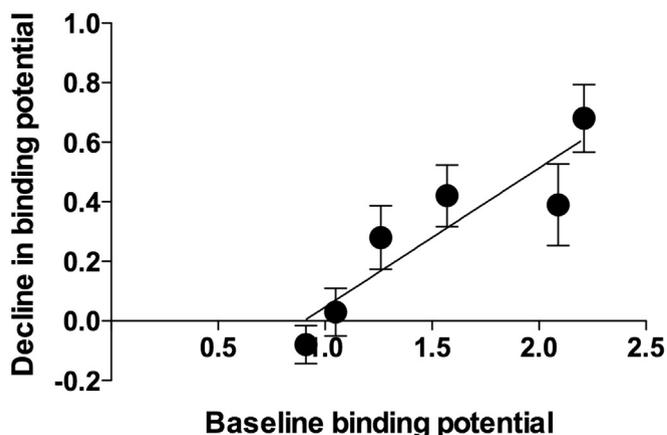


Figure 2. Binding potential change during acute vagal nerve stimulation as a function of baseline binding potentials in six minipig brains. Abscissa: Average binding potential (ratio) of brains at baseline. Ordinate: Average binding potential decline (ratio) from baseline to acute vagal nerve stimulation. Points show average binding potentials of 9 brain regions calculated from volumes of distribution of tracer yohimbine as described in Methods. Error bars represent the standard deviation. Slope of regression line is significantly greater than zero at $P < 0.01$.

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