

RESEARCH PAPER

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Vagal afferent activation decreases brown adipose tissue (BAT) sympathetic nerve activity and BAT thermogenesis*

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ABSTRACT

In urethane/ α -chloralose anesthetized rats, electrical stimulation of cervical vagal afferent fibers inhibited the increases in brown adipose tissue sympathetic nerve activity and brown adipose tissue thermogenesis evoked by cold exposure, by nano-injection of the GABA_A receptor antagonist, bicuculline, in the dorsomedial hypothalamus, and by nano-injection of N-methyl-D-aspartate in the rostral raphe pallidus. Vagus nerve stimulation-evoked inhibition of brown adipose tissue sympathetic nerve activity was prevented by blockade of ionotropic glutamate receptors in the termination site of vagal afferents in the nucleus of the solitary tract, and by nano-injection of GABA_A receptor antagonists in the rostral raphe pallidus. In conclusion, the brown adipose tissue sympathoinhibitory effect of cervical afferent vagal nerve stimulation is mediated by glutamatergic activation of second-order sensory neurons in the nucleus of the solitary tract and by a GABAergic inhibition of brown adipose tissue sympathetic premotor neurons in the rostral raphe pallidus, but does not require GABAergic inhibition of the brown adipose tissue sympathoexcitatory neurons in the dorsomedial hypothalamus.

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Introduction

The levels of brown adipose tissue (BAT) thermogenesis and BAT energy expenditure are regulated by dedicated central neural circuitry that determines BAT sympathetic outflow.¹ The activity in the CNS circuits generating BAT sympathetic nerve activity (SNA) is influenced by peripheral afferent signals, principally those from cutaneous thermal afferents,^{2,3} but also those from other sensors, including arterial chemoreceptors in the carotid sinus nerve, through which hypoxia reduces BAT thermogenesis.⁴ Although visceral sensory endings with afferent axons in the cervical vagus nerve also influence BAT thermogenic responses to febrile^{5,6} as well as metabolic stimuli,^{7–10} our understanding of the roles of vagal afferents in the regulation of BAT and of the central neural pathways through which they regulate BAT SNA are still incomplete.

Largely based on the role of vagal afferent fibers in the regulation of feeding, and on the putative role of the

vagus nerve in the successful weight loss following bariatric surgery, studies have begun to assess the potential of vagal nerve stimulation (VNS) as a treatment for obesity.¹¹ Chronic VNS has been accompanied by weight loss,¹² and increased metabolism in BAT may contribute to such weight loss.¹³ However, other studies have failed to observe weight loss with chronic VNS¹⁴ and mean uptake of ¹⁸F-fluorodeoxyglucose (an index of activation) in human BAT was not increased by VNS.¹⁵ Given the potential role for sympathetic activation of BAT in therapeutic VNS for obesity and the potential for ‘off-target’ metabolic and thermoregulatory effects of VNS used for other indications (such as epilepsy, depression, heart failure, etc.) the current study was designed to determine directly the effect of electrical activation of cervical vagal afferents on rat BAT SNA. Electrical stimulation of cervical vagal afferent fibers inhibits BAT SNA and BAT thermogenesis via glutamatergic activation of neurons in the nucleus of the solitary tract (NTS) and a subsequent GABAergic inhibition of the BAT

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sympathetic premotor neurons in the rostral raphe pallidus area (rRPa).

Materials and methods

All procedures conform to the regulations detailed in the Guide for the Care and Use of Laboratory Animals: Eighth Edition (National Research Council, National Academies Press, 2010) and were approved by the Animal Care and Use Committee of the Oregon Health and Science University.

Male Sprague-Dawley and Wistar rats (344–754 grams, Charles River, Indianapolis, IN, USA) were anesthetized with isoflurane (2–3% in 100% O₂) and the femoral artery and vein were cannulated. No differences in responses were noted between rat strains, therefore the results from both strains were considered as a single group. Rats were then transitioned from isoflurane to a combination of urethane (750 mg/kg, iv) and alpha-chloralose (60 mg/kg, iv) anesthesia. The trachea was cannulated and the animals were artificially ventilated with 100% O₂ (stroke volume: ~1 ml/100 g body weight, 50–70 strokes per minute) and paralyzed with d-tubocurarine (0.6 mg/rat, iv, supplemented thereafter with 0.3 mg when spontaneous respiratory activity was observed). At the time of the tracheal cannulation, the cervical vagi were isolated, and either the left or right vagus was secured to a bipolar hook stimulating electrode with Kwik-sil or Kwik-cast (World Precision Instruments, Sarasota, FL). The stimulated vagus nerve was crushed or transected distal to the electrode and, in most cases, the contralateral cervical vagus was also transected. VNS (1 ms pulses; 80–500 μ A; 1, 2, 5 or 10 Hz) was delivered in 30 s trains at stimulus intensities of 3–4 times the threshold for complete inhibition of BAT SNA at 10 Hz.

Animals were placed in a stereotaxic frame with the incisor bar set to ~4 to 6 mm below the interaural line such that bregma and lambda were level, except for experiments targeting the NTS, in which the incisor bar was positioned –12 mm below the interaural line. Thermocouples were positioned (a) in the rectum to monitor body core temperature (TCORE), which, unless otherwise noted, was maintained at 37.0 \pm 0.5 $^{\circ}$ C with a heat lamp and a water-perfused thermal blanket; (b) in the left interscapular BAT pad to monitor BAT temperature (TBAT); and (c) on the skin of the shaved hindquarter to monitor skin temperature (TSKIN) under the thermal blanket. BAT SNA was

recorded from a sympathetic nerve bundle dissected from the ventral surface of the right interscapular BAT pad and placed on a bipolar hook electrode under warm mineral oil.¹⁶ BAT SNA was differentially amplified (10,000–50,000 times; CyberAmp 380, Axon Instruments, Union City, CA, USA), filtered (1–300 Hz), digitized and recorded onto a hard drive using Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

VNS was applied during increases in BAT SNA produced by a) cooling rats ($n = 8$) with a water-perfused thermal blanket,¹⁷ b) nanoinjection of the GABA_A receptor antagonists, bicuculline (BIC, 500 μ M, 60 nl, $n = 7$)¹⁸ or 2-(3-carboxypropyl)-6-(4-methoxyphenyl)-2,3-dihydropyridazin-3-iminium bromide (GABAzine, 1 mM, 60 nl, $n = 7$) in the rRPa (with a level skull, coordinates were: –3.0 mm caudal to lambda, midline, –9.5–9.8 mm ventral to the dura, or, with the incisor bar at –12 mm below the interaural line and relative to the calamus scriptorius: +3.0 mm rostral, midline, and –2.8 to –3.2 mm ventral), c) nanoinjection of N-methyl-D-aspartate (NMDA; 0.2 mM, 60 nl) in the rRPa ($n = 8$),¹⁶ or d) nanoinjection of BIC (500 μ M, 60 nl) in the dorsal medial hypothalamus (DMH; $n = 5$; with a level skull, coordinates were: –3.0 to –3.3 mm caudal to bregma, 0.5 to 0.7 mm lateral to the midline, and –7.8 to –8.2 mm ventral to dura).¹⁹

Since vagal afferents terminate on second-order sensory neurons in the NTS and use glutamate as their principle excitatory neurotransmitter²⁰ and activation of NTS neurons can inhibit BAT SNA and BAT thermogenesis,²¹ we tested the hypothesis that glutamatergic activation of neurons in the NTS is necessary for the inhibition of BAT SNA evoked by VNS. During increases in BAT SNA evoked by nanoinjection of NMDA in the rRPa ($n = 5$), VNS was performed before and after nanoinjections of the excitatory amino acid receptor antagonists, AP5 combined with CNQX (5 mM each, 80–100 nl) in the NTS (relative to the calamus scriptorius, coordinates were: 0.5 mm rostral, 0.5 mm lateral and 0.5 mm ventral). Control nanoinjections (80–100 nl) of saline vehicle into the NTS were also performed in a subset of rats ($n = 3$).

Data and statistical analyses

All physiological variables and stimulus trigger pulses were digitized (MicroII 1401; Cambridge Electronic

Design, Cambridge, UK) to a computer hard drive for analysis (Spike 2, Cambridge Electronic Design). A continuous measure (4 s bins) of BAT SNA amplitude was obtained from the autospectra of sequential 4-s segments of the raw BAT SNA by calculating the root mean square (rms) value (square root of the total power in the 0.1 to 20 Hz band). The minimal level of BAT SNA was taken as the mean BAT SNA amplitude during a 30 second period recorded either when the rat was in a warm condition (TCORE > 37°C) or following administration of the ganglionic blocker, hexamethonium (30 mg/kg, iv). Percentage inhibitions of BAT SNA were calculated using the formula:

$$1 - \left(\frac{\text{nadir value during stimulation} - \text{control value}}{\text{peak value just prior to vagal stimulation} - \text{control value}} \right)$$

All statistics were performed using Systat software (Version 10, Cranes Software International, Chicago, IL, USA). Data are expressed as mean \pm SE. Statistical comparisons were done between the 30-second period prior to treatment and the 30-second window at the peak or nadir of the treatment-evoked effect. Statistical significance was assessed using paired T-Tests. Results with $p < 0.05$ were considered significant.

Results

VNS inhibits cold-evoked BAT SNA. To test the hypothesis that activation of vagal afferent fibers inhibits the increase in BAT SNA evoked by cold exposure, the cervical vagus nerve was electrically stimulated during cold exposure. During cold

exposure, BAT SNA is characterized by large bursts of activity (Fig. 1A) reflecting the summed action potentials of postganglionic axons innervating BAT. VNS for 30 seconds at frequencies of 2, 5, and 10 Hz significantly inhibited cold-evoked BAT SNA and decreased TBAT by -0.1 ± 0.0 , -0.3 ± 0.1 , and $-0.3 \pm 0.1^\circ\text{C}$, respectively (Figs. 1A, B).

VNS inhibits BAT SNA evoked by disinhibition of neurons in the DMH. Nanoinjection of BIC in the DMH (Fig. 2B) increased BAT SNA to $1127 \pm 259\%$ of control, and VNS delivered during the peak of the BAT activation significantly decreased BAT SNA (Fig. 2A) to $168 \pm 15\%$ of control, a $93 \pm 2\%$ inhibition.

VNS inhibits the increased BAT SNA evoked by nanoinjection of NMDA in the rRPa, but not that evoked by blockade of GABA_A receptors in the rRPa. Nanoinjection of NMDA in the rRPa (Fig. 3D) increased BAT SNA to $2542 \pm 569\%$ of control, and subsequent VNS significantly decreased BAT SNA to $662 \pm 174\%$ of control, representing a $77 \pm 6\%$ inhibition (Fig. 3A). Nanoinjections of BIC (Fig. 3B) and of GABAzine in the rRPa (Fig. 3C) increased BAT SNA to $2371 \pm 726\%$ of control and $2350 \pm 814\%$ of control, respectively. However, subsequent VNS did not affect the amplitude of the increased BAT SNA evoked either by BIC (Fig. 3B, post-drug BAT SNA: $2226 \pm 676\%$ of control, $p = 0.53$) or by GABAzine (post-drug BAT SNA: $2148 \pm 706\%$ of control, $p = 0.33$).

Nanoinjection of glutamate receptor antagonists in the NTS prevents the VNS-evoked inhibition of BAT SNA. Following nanoinjection of saline in the medial NTS (Fig. 4D), nanoinjection of NMDA in the rRPa

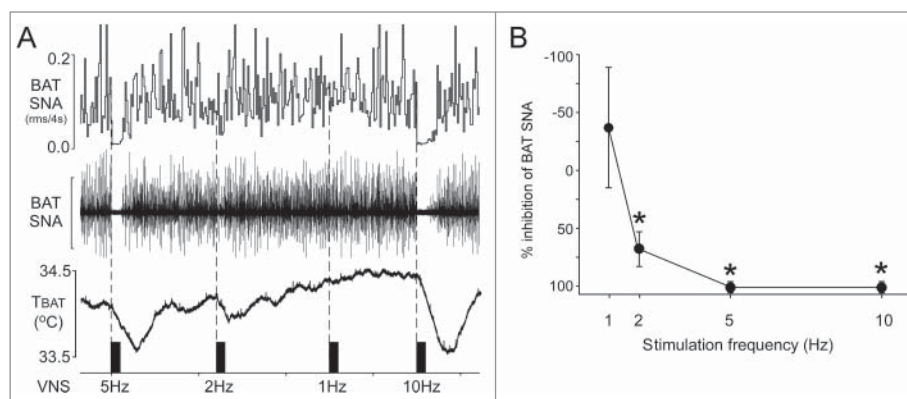


Figure 1. Electrical stimulation of afferent fibers in the cervical vagus nerve inhibits brown adipose tissue (BAT) sympathetic nerve activity (SNA). (A) Representative example demonstrating that vagal afferent fiber stimulation inhibits cold-evoked BAT SNA and BAT thermogenesis. Scale bar for BAT SNA is $200 \mu\text{V}$. (B) Group data (mean \pm SE; $n = 7$) illustrating the frequency response curve of the inhibition of BAT SNA evoked by vagal afferent fiber stimulation. * indicates $p < 0.05$ compared to the pre-stimulation value of cold-evoked BAT SNA. rms, root mean square; TBAT, BAT temperature.

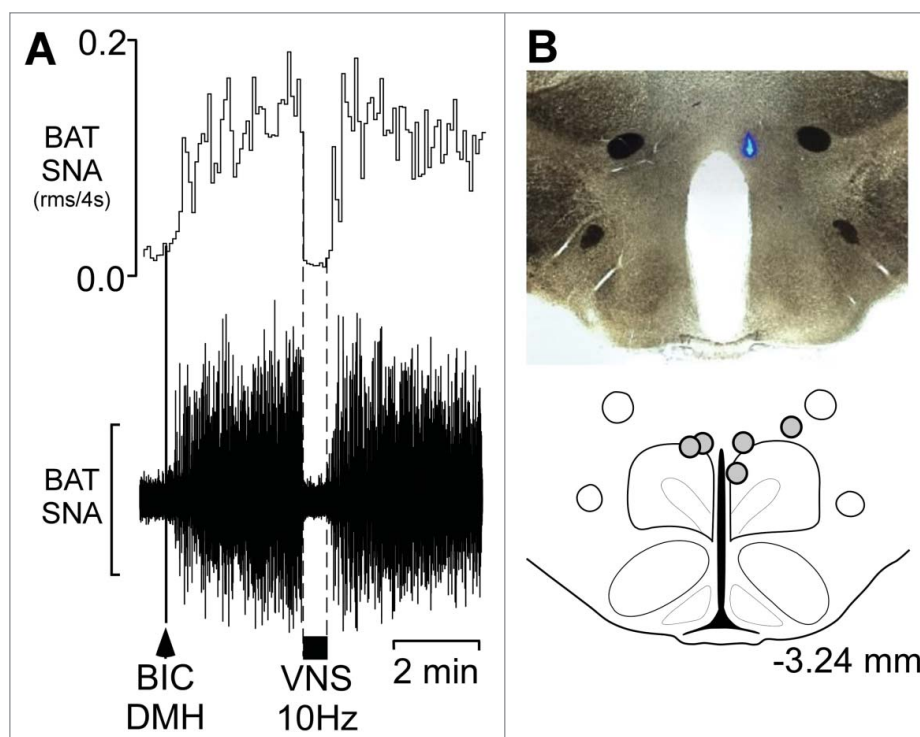


Figure 2. Vagal nerve stimulation (VNS) inhibits the increase in BAT SNA evoked by disinhibition of neurons in the dorsomedial hypothalamus (DMH) (A) Representative example demonstrating that VNS (10 Hz, 1 ms pulses, between the dotted lines) inhibits the increase in BAT SNA evoked by nanoinjection (black arrowhead) of bicuculline (BIC) in the DMH. Scale bar for BAT SNA is $50 \mu\text{V}$. (B) A photomicrograph of a representative nanoinjection site (blue beads) and schematic representation of the BIC nanoinjection sites (filled circles) plotted on an atlas drawing, adapted from,²⁷ approximately 3.24 mm caudal to bregma that includes the DMH.

(Fig. 4C) increased BAT SNA to $1061 \pm 240\%$ of control (Fig. 4A). Subsequent VNS significantly reduced the amplitude of the BAT SNA evoked by nanoinjection of NMDA in rRPa, to $235 \pm 44\%$ of control (Fig. 4A; during VNS: $80 \pm 12\%$ inhibition of BAT SNA), as in naïve rats (Fig. 3A). After nanoinjection of AP5/CNQX in the NTS (Fig. 4D), nanoinjection of NMDA in the rRPa increased BAT SNA to $2411 \pm 1186\%$ of control (Fig. 4B). Subsequent VNS had no effect on the amplitude of the NMDA-evoked level of BAT SNA (Fig. 4B, during VNS: $2299 \pm 1139\%$ of control, $p = 0.38$).

Discussion

Stimulation of afferent fibers in the cervical vagus nerve inhibited the increases in BAT SNA evoked by cold, by disinhibition of neurons in DMH, and by activation of NMDA receptors in the rRPa. In contrast, the increase in BAT SNA evoked by blockade of GABA_A receptors in the rRPa was resistant to inhibition by VNS. These data demonstrate that GABA_A receptor activation in the DMH is not required for the

VNS-evoked inhibition of BAT SNA and suggest that the neural pathway involved in this VNS-evoked inhibition of BAT SNA includes a GABAergic input to the BAT sympathetic premotor neurons in the rRPa. Furthermore, blockade of ionotropic glutamate receptors in the NTS prevented the VNS-evoked inhibition of BAT SNA, an observation consistent with the location of vagal afferent terminals in the NTS and the use of glutamate as the primary neurotransmitter by these afferents.²⁰

The location of the GABAergic neurons whose input to the rRPa is responsible for the vagal afferent-induced inhibition of BAT SNA and BAT thermogenesis remains to be discovered. Although a GABAergic input from the NTS to the BAT sympathetic premotor neurons in the rRPa has been suggested to play a role in the BAT sympathoinhibition evoked from the paraventricular hypothalamus in the mouse,²² in the rat, very few NTS neurons are retrogradely labeled from the rRPa,²³ consistent with the absence of a direct input to the rRPa from the NTS.

VNS has been purported to activate human BAT thermogenesis,¹⁵ although the FDG uptake data

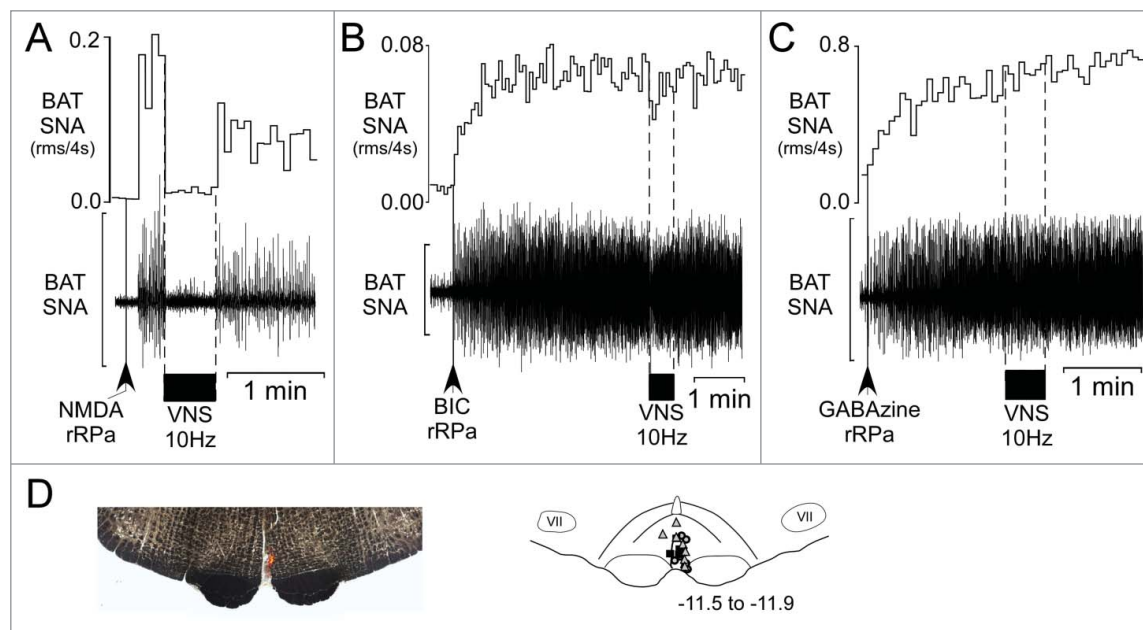


Figure 3. VNS inhibits the increase in BAT SNA evoked by activation of glutamate receptors in the rostral raphe pallidus (rRPa), but not that following blockade of GABA_A receptors in rRPa. (A) Representative example of the VNS-evoked (10 Hz, 1 ms pulses, between the dotted lines) inhibition of the increase in BAT SNA evoked by nanoinjection (black arrowhead) of N-methyl-D-aspartate (NMDA) in the rRPa. (B, C) Representative examples demonstrating that the increases in BAT SNA evoked by nanoinjections (black arrowheads) of the GABA_A receptor antagonists, bicuculline (BIC, B) or GABAzine (C), in the rRPa are not inhibited by VNS (10 Hz, 1 ms pulses, between the dotted lines). Scale bar for BAT SNA is 200 μ V, 50 μ V and 600 μ V in A, B, and C, respectively. (D) Photomicrograph (left) of a representative nanoinjection site (red beads) of GABAzine in rRPa, and an atlas drawing (right), adapted from,²⁷ showing the locations of the nanoinjection sites in rRPa for NMDA (triangles, $n = 7$, the site from one rat was not recovered), BIC (squares, $n = 7$) and GABAzine (circles, $n = 7$). Numbers in lower right indicate the approximate distance from bregma.

reported by Vijgen et al.¹⁵ actually shows that mean BAT activity was not significantly different between periods of active VNS and periods when the vagus nerve was not stimulated. Thus, one must question the apparently erroneous interpretation that BAT thermogenesis played a role in the VNS-evoked increase in energy expenditure in the study of Vijgen et al.¹⁵ Our observation that BAT SNA is inhibited during VNS has important implications for the use of VNS for weight reduction and may help to explain the equivocal effects of VNS on body weight.^{14,24} Reductions in BAT activity during VNS would be expected to reduce overall energy expenditure, thereby counteracting any beneficial effect of the treatment on body weight.

Although the current study indicates that electrical stimulation of vagal afferent fibers elicits a potent sympathoinhibition of BAT, it would be inappropriate to conclude that there are no vagal afferent fibers capable of exciting BAT SNA. At lower frequencies of stimulation (<1 Hz), we have observed VNS-evoked excitatory potentials on BAT SNA and low frequency VNS increased BAT SNA in some rats (Fig. 1).

Furthermore, activations of subpopulations of vagal afferents, such as those containing CCK receptors⁸ or those involved in the early phase of the lipopolysaccharide (LPS)-evoked febrile response can increase thermogenesis,^{5,6} likely via sympathetic activation of BAT.

Our data indicate that the predominant effect of 2–10 Hz electrical stimulation of afferents in the cervical vagus nerve is sympathoinhibitory for BAT SNA, and thus maintaining such VNS would be expected to reduce BAT thermogenesis and BAT energy expenditure. We have not yet identified any endogenous stimuli that might normally activate the relevant vagal afferent fibers that evoke BAT sympathoinhibition. However, we have demonstrated that vagal afferent fibers contribute to the inhibition of cold-evoked activation of BAT SNA and BAT thermogenesis during maintenance on high fat diet,¹⁰ and the activation of these fibers would be expected to contribute to the inhibition of BAT SNA observed during VNS in the current study. Since upregulation of hepatic glucokinase decreases BAT thermogenesis via a vagal afferent mechanism,

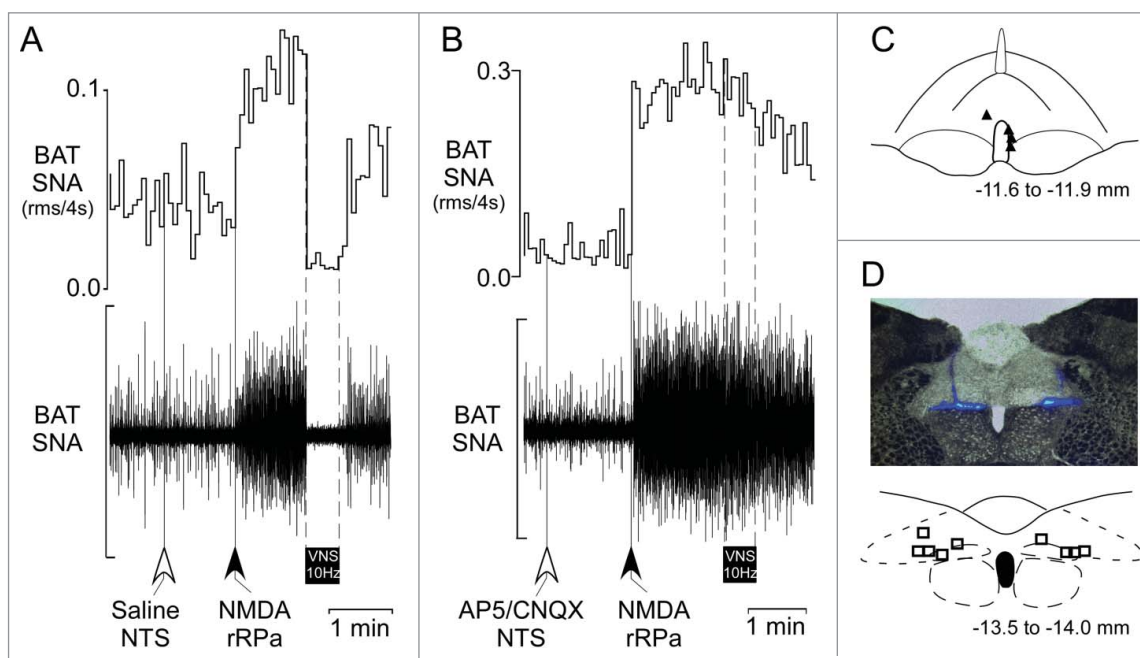


Figure 4. Blockade of glutamate receptors in the nucleus of the solitary tract (NTS) prevents the VNS-evoked inhibition of the increase in BAT SNA evoked by glutamate receptor activation in rRPa. (A) Representative example illustrating the VNS-evoked (10 Hz, 1 ms pulses, between the dotted lines) inhibition of the increase in BAT SNA evoked by nanoinjection (black arrow) of NMDA in the rRPa following nanoinjection (white arrow) of saline in the NTS. (B) Representative example demonstrating the absence of a VNS-evoked (10 Hz, 1 ms pulses, between the dotted lines) inhibition of the increase in BAT SNA evoked by nanoinjection (black arrow) of NMDA in the rRPa after nanoinjection (white arrow) of the glutamate receptor antagonists, AP5/CNQX, in the NTS. (C) Schematic representation of the NMDA nanoinjection sites (solid triangles, one site not recovered) in the rRPa plotted on an atlas drawing, adapted from,²⁷ of a brainstem section at 11.6 to 11.9 mm caudal to bregma, which includes the rRPa. (D) A photomicrograph (upper) of a representative nanoinjection site in the NTS (blue beads) and schematic representation (lower) of the bilateral AP5/CNQX injection sites plotted on an atlas drawing, adapted from,²⁷ of a brainstem section at 13.5 to 14.0 mm caudal to bregma, which includes the medial NTS. Scale bar for BAT SNA is 150 μ V and 300 μ V in A and B, respectively.

hepatic vagal afferents may represent one population of vagal afferents capable of inhibiting BAT SNA. Additionally, intravenous ghrelin suppresses norepinephrine release in BAT via a vagal afferent mechanism,²⁵ although this effect may be concentration dependent.²⁶

In conclusion, activation of a population of vagal afferent fibers is capable of completely inhibiting the sympathetic activation of BAT thermogenesis. The VNS-evoked inhibition of BAT SNA requires glutamatergic activation of neurons in the NTS and is likely mediated via a GABAergic input to the BAT sympathetic premotor neurons in the rRPa. These observations have important implications for the now common, therapeutic applications of VNS.

Abbreviations

AP5 2-amino-5-phosphonopentanoic acid
BAT brown adipose tissue

BIC bicuculline
CNQX 6-cyano-7-nitroquinoxaline-2,3-dione
DMH dorsomedial hypothalamus
GABA gamma-aminobutyric acid
GABAazine 2-(3-carboxypropyl)-6-(4-methoxyphenyl)-2,3-dihydropyridazin-3-iminium bromide
NMDA N-methyl-D-aspartate
NTS nucleus of the solitary tract
rms root mean square
rRPa rostral raphe pallidus area
SNA sympathetic nerve activity
SE Standard error
TBAT brown adipose tissue temperature
TCORE body core temperature
TSKIN skin temperature
VNS vagal nerve stimulation

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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