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Disinhibition of Neurons of the Nucleus of Solitary Tract that Project to the Superior Salivatory Nucleus Causes Choroidal Vasodilation: Implications for Mechanisms Underlying Choroidal Baroregulation

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Abstract

Preganglionic neurons in the superior salivatory nucleus (SSN) that mediate parasympathetic vasodilation of choroidal blood vessels receive a major excitatory input from the baroresponsive part of the nucleus of the solitary tract (NTS). This input appears likely to mediate choroidal vasodilation during systemic hypotension, which prevents decreases in choroidal blood flow (ChBF) due to reduced perfusion pressure. It is uncertain, however, how low blood pressure signals to NTS from the aortic depressor nerve (ADN), which fires at a low rate during systemic hypotension, could yield increased firing in the NTS output to SSN. The simplest hypothesis is that SSN-projecting NTS neurons are under the inhibitory control of ADN-receptive GABAergic NTS neurons. As part of evaluating this hypothesis, we assessed if SSN-projecting NTS neurons, in fact, receive prominent inhibitory input and if blocking GABAergic modulation of them increases ChBF. We found that SSN-projecting NTS neuronal perikarya identified by retrograde labeling are densely coated with GABAergic terminals, but lightly coated with excitatory terminals. We also found that, infusion of the GABA-A receptor antagonist GABAzine into NTS increased ChBF. Our results are consistent with the possibility that low blood pressure signals from the ADN produce vasodilation in choroid by causing diminished activity in ADN-receptive NTS neurons that tonically suppress SSN-projecting NTS neurons.

Keywords

choroidal blood flow; superior salivatory nucleus; nucleus of solitary tract; autonomic; parasympathetic; disinhibition; GABA

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Introduction

Although ChBF was once thought to be unresponsive to changes in perfusion pressure, many studies have now shown that ChBF remains stable over a systemic blood pressure range of about 35% above and below basal levels, with neural mechanisms involved in the compensation (17, 23, 33, 35). When blood pressure is low, choroidal vessels dilate to maintain blood flow to the eye, and when blood pressure is high, choroidal vessels constrict, preventing overperfusion and edema. Neurogenic choroidal vasodilation is mediated by parasympathetic innervation, and neurogenic choroidal vasoconstriction is accomplished by sympathetic innervation. The vasodilation is mediated by the pterygopalatine ganglion (PPG), which innervates orbital vessels feeding the choroidal vessels, as well as choroidal vessels themselves (6, 7, 16, 17, 18, 36, 42), and employs the vasodilators nitric oxide (NO) and vasoactive intestinal polypeptide (VIP).

The preganglionic neurons regulating the PPG reside in the superior salivatory nucleus (SSN), the autonomic component of the facial motor complex (7), and SSN activation increases ChBF (42). In prior studies in rats, we found that a major input to choroidal SSN arises from the nucleus of the solitary tract (NTS) (18, 19). The NTS is organized into viscerotopic subdivisions that receive input selectively from the tongue, vasculature, and thoracic and abdominal viscera (22, 30, 32). Cardiovascular afferents predominantly project to the ipsilateral interstitial and dorsal NTS subnuclei at, and anterior to, the level of the obex (4, 50). Our studies have demonstrated that the part of NTS receiving and/or responsive to baroreceptor information is the source of a projection directly to the ChBF-control neurons of the SSN (18). Since NTS input to SSN is excitatory (1, 2, 18) and NTS activation increases ChBF (20), the NTS input to SSN may mediate choroidal vasodilation during systemic hypotension. It is unclear, however, how low blood pressure signals to NTS from the aortic depressor nerve (ADN), which has excitatory input to NTS (21) and fires at a low rate during systemic hypotension (49), could yield increased firing in the output of NTS to SSN, and ultimately to choroidal vasodilation. One possibility is that SSN-projecting NTS neurons receive innervation from inhibitory ADN-receptive NTS neurons (Fig. 1). During normal or high systemic blood pressure, the SSN-projecting NTS neurons would be inhibited by these ADN-receptive NTS neurons. When blood pressure is low, however, the ADN-receptive inhibitory NTS neurons would fire at a low rate, with the SSN-projecting NTS neurons then being disinhibited. Consistent with this possibility, GABAergic neurons are present in baroreceptive NTS (13), and the distribution of neurons enriched in GABA-A receptors in NTS overlaps the distribution of SSN-projecting NTS neurons (38, 45, 46). In the present study, we provide both morphological and physiological evidence to support this framework for blood pressure-dependent control of ChBF.

Methods

Animal Preparation

Adult male Sprague-Dawley (SD) rats (290g–380g; from Harlan, Indianapolis, IN) were used in either neuroanatomical studies to characterize inhibitory versus excitatory inputs to SSN-projecting NTS neurons (n=3), or in physiological studies to assess the effects of a GABA-A antagonist infused into NTS on ChBF (n=5). All experiments were undertaken in

compliance with the ARVO statement on the Use of Animals in Ophthalmic and Vision Research, and with NIH guidelines and institutional approval.

Retrograde tracer injection into SSN

To identify SSN-projecting neurons in NTS, we made injections of BDA3K into SSN by iontophoresis, as described previously (18, 19). Animals were allowed to survive 10–14 days, and were then anesthetized, and perfused transcardially with 150 – 200ml sodium phosphate buffered saline (PBS, 0.85% sodium chloride dissolved in 0.01M PB) followed by 400–500 ml 4% paraformaldehyde prepared in 0.1M sodium phosphate buffer (pH7.4) with 0.1M lysine and 0.01M sodium periodate (PLP fixative). Brains were removed, cryoprotected at 4°C in 20% sucrose - 10% glycerol - 0.138% sodium azide in 0.1M sodium phosphate buffer, frozen, and sectioned at 40 µm in the transverse plane with a sliding microtome. Sections were stored at 4°C in a 0.02% sodium azide - 0.02% imidazole in 0.1 M PB until processed for detection of VGLUT2-containing and GAD67-containing terminals on the BDA3K-containing SSN-projecting NTS neurons, as described below. Note that VGLUT2 is enriched in baroreceptive afferents ending in NTS (21).

Multiple-label fluorescence immunohistochemistry

We used immunofluorescence multiple labeling to visualize VGLUT2+ and GAD65+ terminals and BDA3K+ neurons in NTS. The sections were rinsed and incubated in streptavidin Alexa-488 (1:200; Molecular Probes) for 24 hours at 4°C to label the BDA3K+ SSN-projecting neurons in NTS. The sections were then rinsed and incubated in mouse anti-GAD65 (Sigma-Aldrich, 1:200) and either guinea pig anti-VGLUT2 (Millipore, 1:1000) or rabbit anti-VGLUT2 (Sigma-Aldrich, 1:1000), for 24 hours at 4°C. The antibodies were diluted with 0.1M PB – 0.8% Triton X-100. After rinsing, the tissue was incubated in donkey anti-mouse IgG-Alexa-594 (1:200; Molecular Probes), and either goat anti-guinea pig IgG-Alexa-647 or donkey anti-rabbit IgG-Alexa-647 (1:200; Molecular Probes), overnight at 4°C. The sections were then mounted on gelatin-coated slides, air-dried, and cover-slipped in glycerol phosphate buffer (9:1). Sections were viewed and images captured using a Zeiss 710 confocal laser-scanning microscope.

Choroidal Blood Flow Measurements

For ChBF studies, rats were anesthetized by ip injection of 0.1 ml/100 g of ketamine/xylazine (87/13 mg/kg), with supplemental doses (0.05–0.07 ml of ketamine/xylazine mixture) every 25–30 min. To measure systemic arterial blood pressure (ABP), the femoral artery was catheterized. A Digi-Med Blood Pressure Analyzer™ (BPA-100, Micro-Med, Inc., Louisville, KY) was used to measure ABP via a pressure transducer connected to the femoral artery cannula. The rats were then positioned in a stereotaxic device. A pulse oximeter on the tail or hind foot measured systemic blood oxygen saturation. Body temperature was maintained at 37°C with a Harvard heating blanket and rectal thermoprobe.

To prepare the eye for transcleral measurement of ChBF, fascia overlying the superior pole of the right eye and the Harderian gland was removed. The tip of a laser Doppler probe connected to a LASERFLO BPM² blood perfusion monitor (Vasamedics; Eden Prairie, MN) was positioned 1–3mm above the sclera with a micromanipulator. Aquasonic ultrasound gel

(Parker Laboratories, Inc.; Fairfield, NJ) or 33% glycerol – 0.1M sodium phosphate buffer (PB) was used in the interface between the probe tip and the sclera to prevent tissue drying during the experiment. Measurements of ChBF were made from the vascular bed beneath the sclera in the gap between the superior and medial rectus muscles. Our previous work has shown that relative ChBF can reliably be measured transclerally using Laser Doppler Flowmetry (11, 12, 13, 33). The LASERFLO BPM² data was exported to a ML880 PowerLab 16/30 data acquisition system (AD Instruments Inc., Colorado Springs, CO), and the digitized data were collected and analyzed using LabChart v7 Pro software (AD Instruments Inc). The data averaging time window of the BPM² was set at 0.3 sec, with the sampling rate on PowerLab set at 200 samples/sec. For placement of a micropipet in NTS, the skin over the skull was retracted and bone over NTS removed. The rat brain stereotaxic atlas of Paxinos and Watson (31) was used for coordinates. The glass pipet for iontophoretic delivery (n=3) was prepared by filling the tip with saline and then backfilling with 5 mM GABAzine dissolved in water. To determine that the tip of the glass electrode was within choroid-responsive NTS, negative current pulses trains of 100 Hz, 0.5 msec, 7 sec duration and –20 μ A amplitude were used to elicit a ChBF increase (10, 12, 41). Before iontophoresis with positive current (7s on, 7s off, 5 μ A, 10 min duration) was applied, a negative holding current was used to prevent unwanted leakage of GABAzine into NTS. In two additional rats, 500 μ M GABAzine was pressured injected into NTS using a Hamilton microsyringe with glass micropipet attached using a compression-fitting adaptor, to obviate concerns about current-driven responses during iontophoretic delivery of GABAzine.

Histological Analysis of ChBF Cases

After the ChBF recording, rats were perfused transcardially as described above, and brains were sectioned frozen, using a sliding microtome. Evaluation of pipet tip placement within NTS was based on detection of the electrode tip in NTS in cresyl violet-stained sections, or detection of neurons in NTS activated by GABAzine in c-fos immunostained section. In some cases, the c-fos immunostaining was combined with parvalbumin immunolabeling using two-color DAB processing, because the paucity of parvalbumin in NTS helps delineate it. Our immunolabeling and cresyl violet staining procedures have been described in detail in our previous publications (9, 18, 19).

Statistical analysis

We used t-test to compare excitatory and inhibitory input to the soma and proximal dendrites of SSN projecting NTS neurons. We used paired t-tests to assess the impact of GABAzine injected into NTS on ChBF and ABP.

Results

GABAergic Terminals on SSN-projecting NTS Neurons

SSN-projecting NTS neurons were retrogradely labeled by iontophoretic injection of BDA3K into SSN, and sections triple-labeled to detect the BDA3K+ NTS neurons and their GABAergic (GAD65+) and glutamatergic (VGLUT2+) inputs. Numerous neurons well labeled with BDA3K were seen in NTS in 3 rats, with a distribution as described in our prior studies (18, 19). The multiple labeling showed that contacts by GAD65+ terminals were

much more common on the BDA3K+ NTS neurons than were contacts by VGLUT2+ terminals, particularly on the cell bodies of the labeled NTS neurons (Fig. 2). Measurements of the abundance of these two terminal types for 18 BDA3K+ NTS neurons showed that individual GAD65+ terminals were significantly more numerous (about 4×) on the soma of the BDA3K+ NTS neurons than were individual contacts by VGLUT2+ terminals ($p=0.0000022$). Similarly, GAD65+ terminals covered significantly more (about 6×) of the soma surface for the BDA3K+ NTS neurons (Fig. 3) than did contacts by VGLUT2+ terminals ($p=0.0000021$). GAD65+ terminals and VGLUT2+ terminals were, however, relatively comparable in their abundance on and areal coverage of the proximal dendrites of BDA3K+ NTS neurons (Fig. 3) ($n=9$ neurons).

Disinhibition of SSN-projecting NTS Neurons with GABAzine

Accurate placement of the pipet tip in NTS was obtained in five rats, as shown by subsequent histological evaluation, with multiple GABAzine delivery trials in the case of one rat, using positive current for iontophoretic delivery. The iontophoretic infusion of 5 mM GABAzine led to an increase in ChBF, typically with a latency of 6.42 minutes and with a mean duration of 22.4 minutes after onset of the increase (Fig. 4A). The prolonged latencies and durations are likely to represent the time needed for enough GABAzine to diffuse to and away from, respectively, SSN-projecting NTS neurons, and to then have an impact on ChBF. Moreover, the delay indicates that the current itself did not provoke the response, since in the studies performed here, as well as in other studies of ours (20), we have found that ChBF response to cathodal current pulses delivered to NTS are brisk (latency less than a 500 msec) (Fig. 4B). In addition, they require larger current amplitudes (20 μ A) than used here for iontophoresis (5 μ A). The ChBF increases after GABAzine delivery were not accompanied by significant changes in ABP (Fig. 4A). By contrast, ChBF and ABP remained stable prior to GABAzine delivery, nor were any changes observed during negative iontophoretic current delivery. Pressure injection of 500 μ M GABAzine also led to increases in ChBF. The results of iontophoretic and pressure delivery were grouped for statistical analysis of ChBF and ABP changes, with the various GABAzine trials pooled and compared to behavior during the baseline period prior to GABAzine delivery. Mean ChBF was significantly increased over baseline by 41.1% ($p=0.0053$) (Fig. 5). By contrast, mean ABP was not significantly changed (−4.1%, $p=0.3530$) (Fig. 5).

Discussion

The parts of NTS projecting to the choroidal neurons of SSN receive ADN baroreceptive input via the vagus nerve and/or respond to systemic ABP fluctuation (4, 14, 20, 25, 37). Increases in blood pressure yield increases in firing in the ADN and its target NTS neurons, and decreased ABP reduces firing in ADN and its target NTS neurons (49). Our studies have demonstrated that the part of NTS receiving and/or responsive to baroreceptor information, in particular, is the source of a projection directly to the ChBF-control neurons of the SSN (18). Since NTS input to SSN is excitatory (1, 2, 18) and NTS activation increases ChBF (20), the NTS input to SSN is likely to mediate choroidal vasodilation during systemic hypotension. It has been uncertain, however, how low blood pressure signals to NTS from the ADN could yield increased firing in the NTS output to SSN, since the ADN fires at a

low rate during systemic hypotension. The simplest hypothesis is that SSN-projecting NTS neurons receive input from inhibitory ADN-receptive NTS neurons (Fig. 1). Consistent with this hypothesis, we show here that SSN-projecting NTS neurons are under prominent inhibitory control, since the perikarya of SSN-projecting NTS neurons identified by retrograde labeling are densely coated with GAD65+ terminals, but comparatively lightly coated with excitatory VGLUT2+ terminals. In this regard, they resemble neurons of globus pallidus internus, which are under prominent inhibitory control via input from GABAergic striatal projection neurons, which interweaves with a lesser excitatory input from the subthalamic nucleus (3, 15). We also show that infusion of the GABA-A receptor antagonist GABAzine into NTS increases ChBF, consistent with our morphological evidence that the neurons in NTS that regulate ChBF are under inhibitory control. Our results are thus consistent with the possibility that low ABP signals from the ADN can cause a vasodilation in choroid by means of diminished ADN drive to GABAergic inhibitory NTS neurons that exert tonic inhibition of spontaneously active SSN-projecting NTS neurons (Fig. 1). This possibility is supported by the findings that NTS neurons receiving polysynaptic input from baroreceptive afferents respond with diminished firing in response to ADN activation (39) and increased firing in response to reductions in ABP (49), that GABAergic NTS neurons are activated by hypertension (47), and that GABA-responsive NTS neurons with ascending projections show diminished firing during hypotension (44). To further confirm our hypothesis, it would be useful to demonstrate that SSN-projecting NTS neurons, in particular, show activity changes opposite to the direction of ABP changes.

The baroreceptive part of NTS is also involved in regulation of systemic ABP by a multisynaptic output to sympathetic preganglionic neurons. High systemic ABP signals cause diminished sympathetic vasoconstrictory drive that produce vasorelaxation, which, in turn, reduces ABP. In this case as well, the sign of the input to NTS must be reversed in the brainstem outflow signal to the sympathetic preganglionic neurons of the spinal cord. To achieve this, baroreceptive NTS has an excitatory projection to GABAergic neurons of the caudal ventrolateral medulla (CVLM), which, in turn, project to excitatory neurons of the rostral ventrolateral medulla (RVLM) that project to sympathetic preganglionic neurons of the spinal cord (40). Activation of neurons in the baroreceptive NTS by heightened ABP thus leads to activation of CVLM via NTS, leading to increased inhibition of RVLM, and diminished excitatory outflow from RVLM to sympathetic preganglionic neurons (14). This reduces systemic vasoconstriction, which serves to combat the heightened ABP and mediates one part of the systemic baroreflex (14). The present results show that NTS neurons projecting to CLVM, unlike those projecting to SSN, do not appear to be under prominent inhibitory control (since GABAzine into NTS did not significantly affect ABP), but rather appear to be under the excitatory control of the baroreceptive ADN input (5, 8, 43).

Impairments in parasympathetic control of ChBF can adversely affect retinal health (12, 34, 36). By showing that NTS neurons that drive ChBF are under prominent GABA-A receptor-mediated inhibitory control, our findings suggest that drugs that act as agonists at GABA-A receptors could reduce ChBF and thereby potentially adversely affect the outer retina and macula. This could, in fact, help explain instances in which sustained high-dose long-term treatment with benzodiazepines has been associated with macular degeneration (24, 26).

Moreover, as cerebral blood flow appears to be baroregulated by the same circuit as ChBF (1, 2, 18, 19), our findings may also explain why high doses of benzodiazepines reduce cerebral blood flow and alter its baroregulation (27, 28, 29, 48).

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Abbreviations

Ach	acetylcholine
ADN	aortic depressor nerve
ABP	arterial blood pressure
ChBF	choroidal blood flow
ChBVol	choroidal blood volume
ChBVel	choroidal blood velocity
CVLM	caudal ventrolateral medulla
Glu	glutamate
GAD	glutamic acid decarboxylase
NO	nitric oxide
NOS	nitric oxide synthase
NTS	nucleus of solitary tract
RVLM	rostral ventrolateral medulla
SSN	superior salivatory nucleus
VIP	vasoactive intestinal polypeptide
VGLUT2	vesicular glutamate transporter 2

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Highlights

1. NTS neurons controlling choroidal blood flow receive dense inhibitory input
2. Disinhibition of NTS neurons causes increases in choroidal blood flow
3. Disinhibition of NTS during low blood pressure may drive choroidal vasodilation
4. Sign inversion of a low blood pressure signal may drive choroidal baroregulation

Figure 1.

Schematic illustrating the hypothetical circuit by which baroreceptive input to the NTS may mediate disinhibitory control of choroidal vasodilation via the SSN.

Figure 2.

Images showing the greater abundance of GAD65+ inhibitory terminals on SSN-projecting NTS neuron perikarya than VGLUT2+ excitatory terminals. The SSN-projecting neurons shown in the A–B and C–D image pairs were retrogradely labeled from SSN and visualized using streptavidin Alexa-488, while GAD65 was visualized with Alexa-594 and VGLUT was visualized with Alexa-647. Images were captured with a CLSM, and are displayed in these image pairs with the GAD65 labeling (red) shown together with the labeling of SSN-projecting NTS neurons (green) in images A and C, and the VGLUT2 labeling (displayed here as red) shown together with the labeling of SSN-projecting NTS neurons (green) in images B and D.

Figure 3.

Bar graph illustrating that GAD65+ terminals covered significantly more of the soma of the BDA3K+ NTS neurons than did contacts by VGLUT2+ terminals, but GAD65+ terminals and VGLUT2+ terminals were relatively comparable in their areal coverage of the proximal dendrites of BDA3K+ NTS neurons. Asterisk indicates a significant difference between GAD65+ and VGLUT2+ terminals.

Figure 4.

Chart recordings showing that iontophoretic GABA_Azine infusion into NTS (A) produced a sustained increase in ChBF, but not ABP (C). The response had a latency of about 600 seconds from stimulus onset (arrow) and then lasted about 1500 seconds beyond stimulus offset (arrow). In contrast to iontophoretic GABA_Azine infusion with 7 second on – 7 second off 5 μ A cathodal pulses, 7 second trains of 0.5 msec 100 Hz 20 μ A cathodal pulses produced rapid and brief increases in ChBF (B). Note that in both B and C, the x-axis shows seconds. The site of GABA_Azine infusion is shown by the asterisk at the center of the c-fos positive labeling of nuclei within NTS, whose borders are outlined (A). The c-fos nuclear labeling reflects neurons activated by the GABA_Azine infusion.

Effect of GABAzine Injection into NTS on ABP and Choroid

Figure 5.

Bar graph illustrating that GABAzine infusion into NTS produced increases in ChBF, but not ABP. Asterisk indicates a significant increase above baseline after GABAzine administration