

**Background:** Increasing evidence indicates that GABAergic neurons in the nucleus of the solitary tract (NTS) play a significant role in the arterial baroreceptor reflex and control of cardiovascular homeostasis. However, the role of these neurons in the development of hypertension is not yet fully clear.

**Methods and Results:** In the present study, we first confirmed that GABA\(_B\) receptor (GBR) expression is enhanced in the NTS of SHR as compared with WKY rats using real-time RT-PCR and western blots. To study the functional consequence of upregulated GBR expression, GBR was overexpressed in the NTS by bilateral microinjection of the AAV2-GBR1 viral vector into the NTS of WKY rats. Immunofluorescence staining and western blots demonstrated that microinjection of AAV2-GBR1 into the NTS of WKY rats resulted in a significant increase in GBR1 expression in the NTS neurons. Overexpression of GBR in the NTS induced a chronic elevation in blood pressure and heart rate in the normotensive WKY rats. In an acute study, the pressor response to baclofen microinjected into the NTS was enhanced in SHR as compared with WKY rats.

**Conclusions:** GBR1 expression is enhanced in the NTS of SHR vs. WKY rats and overexpression of this gene in the NTS results in chronic elevation of blood pressure and heart rate in normotensive rats. (Circ J 2013; 77: 2558–2566)

**Key Words:** Blood pressure; GABA\(_B\) receptor; Hypertension

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**GABA\(_B\) Receptor Gene Transfer Into the Nucleus Tractus Solitarii Induces Chronic Blood Pressure Elevation in Normotensive Rats**

Bo Li, MD; Qing Liu, BSc; Chengluan Xuan, MD; Lirong Guo, PhD; Ruofan Shi, BSc; Qi Zhang, PhD; Stephen T. O’Rourke, PhD; Kexiang Liu, MD; Chengwen Sun, PhD

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The nucleus tractus solitarius (NTS) is a termination site for primary afferent fibers from baroreceptors, and other peripheral cardiovascular receptors, that contain blood pressure (BP)-sensitive neurons. As such, it mediates the inhibitory actions of baroreceptors on sympathetic discharge.\(^1\) The intermediate portion of the NTS is richly innervated by fibers from various brain nuclei that are known to have an important role in cardiovascular control, including the area postrema and hypothalamic nuclei.\(^2\) Thus, it is believed that the NTS plays an important role in BP regulation, as well as contributing to the development and maintenance of hypertension. Several studies have demonstrated that the commissural NTS may be altered in the spontaneous hypertensive rat (SHR).\(^3\) These cardiovascular regulatory actions are carried out by several neuronal transmitters and modulators [eg, such as \(\gamma\)-aminobutyric acid (GABA)].\(^4\)

GABA is a well-known neurotransmitter that exerts inhibitory actions in the brain, mediated through its receptors, which include the ionotropic GABA\(_A\) receptor (GAR) and metabotropic GABA\(_B\) receptor (GBR), that are defined on the basis of pharmacological and physiological studies.\(^5\) The ionotropic GAR has an intrinsic \(\text{Cl}^-\) channel, which is responsible for inducing fast inhibitory postsynaptic potentials. The GBR is a G-protein-coupled receptor, and regulates neuronal activity via activation of \(\text{K}^+\) channels, which in turn induces hyperpolarization of the neuronal membrane and chronic inhibition of neuronal activity. A high density of both GAR and GBR and a high density of GABA-containing nerve terminals have been found within the NTS.\(^6\) The GBR is a heterodimer comprised of GBR1 and GBR2 proteins. GBR1 is critical for ligand activation of the heterodimer, whereas GBR2 is merely a shuttle protein to transfer GBR1 onto the surface of the cell mem-
GABA B Receptor in NTS

Recording of Arterial Pressure and Sympathetic Nerve Activity

Chronic BP recording was carried out with a radiotelemetry system as described previously. Continuous recordings were started 5 days after transducer implantation, in order to allow the animals to recover from the surgical procedure.

Acute BP and heart rate (HR) recordings were carried out using PE-10 catheters fused to PE-50 catheters and performed under urethane (1 g/kg, IP) anesthesia. The catheter was perfused with heparinized saline (100 IU/ml), placed in the right femoral artery, and connected to a BP transducer and a bridge amplifier (AD Instrument, Colorado Springs, CO, USA). The BP and HR data were collected and analyzed with Powerlab software (AD Instrument, Colorado Springs). Renal sympathetic nerve activity (RSNA) was recorded in SHR and WKY rats, before and after NTS injection, as described previously.

NTS Microinjection and GBR1 Gene Transfer Into the NTS

Agonists or antagonists of GBR or GAR were bilaterally microinjected into the NTS of SHR and WKY rats, according to procedures described previously. For GBR1 gene transfer into the NTS, WKY rats were anesthetized with a mixture of O2 and isoflurane. The adeno-associated virus type 2 (AAV2) containing the rat GBR1a gene (AAV2-GBR1), or green fluorescent protein (AAV2-GFP) as control, driven by a chicken β-actin promotor with a human cytomegalovirus enhancer, was used to induce endogenous GBR1 expression in the NTS. The AAV2-GBR1 and AAV2-GFP plasmids were constructed and prepared as described previously, and each (all in 1×10⁹ gc in 50 nl) was microinjected bilaterally into the NTS as described previously.

Real-Time RT-PCR

Real-time PCR was used to detect changes in the expression of GAR and GBR in the NTS of SHR rats and WKY rats. Isolation of total RNA from NTS tissue, reverse transcription, and
the specific probes for GBR1 and GBR2 were described in our previous study. In each experiment, samples were analyzed in triplicate.

**Western Blot Analysis**

GBR1 and GARγ2 protein levels in rat brain sections of the NTS were assessed by western blot analysis as described previously.

**Immunohistochemistry**

Immunofluorescence staining of brain NTS sections was performed as described previously. In brief, the NTS sections, identified with a rat brain atlas, were incubated for 60 min with phosphate-buffered saline plus 0.5% Tween 20 (PBS-T) containing 5% goat serum. Slices were incubated with primary antibodies (anti-NeuN monoclonal antibody, 1:500; rabbit anti-GFP, 1:500) overnight at 4°C. After being washed with PBS-T, the sections were incubated with secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG, 1:1,000; Alexa Fluor 594 goat anti-mouse IgG, 1:1,000) for 2 h. The sections were then washed with PBS-T, and examined under a confocal fluorescence microscope (Olympus, Fluoview FV300). The fluorescent images were collected and analyzed with flow-view software.

**Statistical Analysis**

All data are presented as means±SE. Statistical significance was evaluated by 1- or 2-way ANOVA, as appropriate, followed by either a Newman-Keuls or Bonferroni post hoc analysis, where appropriate. Differences were considered significant at P<0.05, and individual probability values are noted in the figure legends.

**Results**

**GAR and GBR Expression in the NTS of SHR vs. WKY Rats**

The aim of our first experiment was to confirm whether GBR expression is increased in the NTS of SHR. GBR1 and GBR2 mRNA levels in the NTS of SHR and WKY rats were detected with real-time RT-PCR. The results shown in Figure 1A demonstrate that both GBR1 and GBR2 mRNA levels in the NTS of SHR were significantly higher than those of WKY rats; however, GBR1 and GBR2 mRNA levels in the PVN were comparable between SHR and WKY rats. For comparison, we also determined GAR expression in the NTS of SHR and WKY rats. The data shown in Figure 1B indicate that both GARα1 and GARγ2 are expressed in the NTS of SHR and WKY rats and that the mRNA levels of GARα1 and GARγ2 in the NTS were comparable between the 2 strains of rats.

GBR1 and GARγ2 protein levels in the NTS were determined by western blot analysis in SHR and WKY rats. The results shown (Figure 2A) indicated that GBR1 protein levels were enhanced more than 2-fold in SHR, as compared with WKY rats. In contrast, GARγ2 expression was comparable between SHR and WKY rats (Figure 2B). These results demonstrated that GBR1 expression in NTS was enhanced in the NTS of SHR and that GARγ2 expression was not significantly altered.

![Figure 2](image-url)
between SHR and WKY rats.

Overexpression of GBR1 in the NTS by Gene Transfer
To study the functional consequence of enhanced GBR1 expression in the NTS of SHR, and whether enhanced GBR1 expression in the NTS could result in an elevation of BP, *GBR1* was overexpressed using AAV2 viral vector-mediated gene transfer (AAV2-GBR1) into the NTS of normotensive WKY rats. The results presented in Figure 3 show immunofluorescence of GFP expression in the NTS on the 7th day after microinjection of AAV2-GFP. Figures 3A–C shows a high magnification view indicating that GFP expression is localized to neurons of the NTS. Figure 3D and Figure 3E demonstrate a strong immunoreactive signal in the NTS after transfection. These results indicated that the microinjection of AAV2-GFP significantly increased *GFP* expression in the neurons within the NTS.

We next examined GBR1 mRNA levels in the NTS at the time points indicated in Figure 4A after microinjection of AAV2-GBR1 or AAV2-GFP into the NTS. GBR1 mRNA levels in the NTS were significantly increased by AAV2-GBR1 gene transfer (Figure 4A). The increased *GBR1* expression started on day 1, reached a peak in 2 days, and lasted at least for 14 days after NTS injection of the viral vector. In contrast, microinjection of AAV2-GFP did not alter *GBR1* expression in the NTS. GBR1a protein levels were examined in the NTS after microinjection of AAV2-GBR1 and AAV2-GFP. GBR1a protein levels were significantly enhanced in the NTS after gene transfer (Figure 4B), as compared with control rats, which received NTS microinjection of AAV-GFP. The results indicated that AAV2-mediated GBR1 gene transfer induced a significant increase in GBR1 protein expression. However, microinjection of AAV2-GFP control vector intro the NTS did not alter GBR1 protein levels (data not shown).

Effect of GBR1 Gene Transfer on Arterial Pressure, HR, and Norepinephrine Plasma Levels
Based on the expression data, we determined whether increased expression of GBR1 in the NTS of normotensive rats would alter basal BP in normotensive rats. At 1 week after implantation of the BP, gene transfer (AAV2-GBR1 and AAV2-GFP). GBR1a protein levels were significantly enhanced in the NTS after gene transfer (Figure 4B), as compared with control rats, which received NTS microinjection of AAV-GFP. The results indicated that AAV2-mediated GBR1 gene transfer induced a significant increase in GBR1 protein expression. However, microinjection of AAV2-GFP control vector intro the NTS did not alter GBR1 protein levels (data not shown).
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NTS injection of AAV2-GFP did not alter plasma NE levels (288±25 pg/ml in control rats; 301±32 pg/ml and 292±31 pg/ml in rats at 7 and 14 days after NTS injection of AAV2-GFP, n=4 rats in each group, P>0.05). These results indicated that overexpression of GBR1 in the NTS elevated the plasma level of NE, which is a marker of chronic SNA.

**Effect of Agonists and Antagonists of GBR and GAR on BP, HR, and RSNA in SHR and WKY Rats**

The experimental results just described indicates that GBR1 expression was significantly enhanced in the NTS of SHR rats. To examine whether these enhanced GBR1 are functional in the regulation of BP, we further investigated the effects of agonists and antagonists of GBR and GAR on BP and HR in SHR and WKY rats.
SHR and WKY rats (Figure 5F). In addition, microinjection of an equal volume of saline at the same site in the NTS did not alter MAP and HR in either strain of rat (data not shown). These data indicated that the effect of muscimol microinjected into the NTS on BP and HR was comparable between WKY and SHR rats.

The effect of the GAR antagonist, bicuculline, microinjected into the NTS on BP and HR was also examined in SHR and WKY rats. Microinjection of bicuculline (10 pmol in 50 nl) into the NTS resulted in a significant decrease in MAP from 110±7 to 89±5 mmHg (n=6, P<0.05) in SHR and from 84±5 to 62±4 mmHg (n=5, P<0.05) in WKY rats (Figure 5C). However, the depressor response to bicuculline microinjected into the NTS did not differ between SHR and WKY rats (Figure 5E).

HR in both SHR and WKY rats was significantly reduced from 338±12 to 308±13 beats/min (n=4, P<0.05) and from 332±17 to 308±13 beats/min (n=6, P<0.05) by muscimol NTS injection (Figure 5B). However, the HR increases evoked by microinjection of muscimol into the NTS were comparable between SHR and WKY rats (Figure 5E).
to 295±6 beats/min (n=5, P<0.05), respectively, before and after NTS administration of bicuculline (Figure 5D). The HR response evoked by bicuculline was comparable between SHR and WKY rats (Figure 5F). These results demonstrated that the depressor response to the GAR antagonist, bicuculline, microinjected into the NTS was comparable between SHR and WKY rats.

In another group of rats, we examined the effect of micro-injection of a selective GBR agonist, baclofen, into the NTS of SHR and WKY rats. Microinjection of baclofen (50 pmol, 50 nl) into the NTS caused BP increases in both SHR (from 112±8 to 150±7 mmHg, n=6, P<0.01) and WKY rats (from 87±5 to 108±7 mmHg, n=6, P<0.05) (Figure 6A). The pressor response to baclofen microinjected into the NTS was significantly increased in SHR as compared with WKY rats (Figure 6E). In addition, NTS administration of baclofen also increased the HR in SHR (from 302±17 to 375±17 beats/min, n=6, P<0.05) and WKY rats (from 282±14 to 317±14 beats/min, n=6, P<0.05) (Figure 6B). The tachycardic response to baclofen was significantly increased in SHR as compared with WKY rats (n=6, P<0.05) (Figure 6F). In addition, RSNA was recorded in SHR and WKY rats before and after NTS micro-injection of baclofen (50 pmol, 50 nl). Microinjection of baclofen into the NTS increased RSNA by 21±3% and 13±2% in SHR and WKY rats, respectively. The sympathoexcitatory effect of baclofen was significantly enhanced in SHR (n=4 rats in each group, P<0.05) as compared with WKY rats. In summary, these data indicated that both the pressor and sympathoexcitatory effects of baclofen in the NTS were enhanced in SHR as compared with WKY rats.

Figure 6. Effect of the GBR antagonist, CGP35348, and the agonist, baclofen, on MAP and HR in SHR or WKY rats. (A, B) Time course data showing the MAP (A) and HR (B) changes evoked by baclofen (50 pmol in 50 nl) microinjected into the NTS of SHR (filled dots) and WKY rats (open dots). Data are mean±SE (n=6 rats in each group). *P<0.05 or **P<0.01 as compared with respective basal condition. (C, D) Time course data showing the MAP (C) and HR (D) changes evoked by CGP35348 (100 pmol in 50 nl) microinjected into the NTS of SHR (filled dots) and WKY rats (open dots). Data are mean±SE (n=6 rats in each group). *P<0.05 or **P<0.01 as compared with respective basal condition. (E, F) Bar graphs showing MAP (E) and HR (F) changes evoked by microinjection of baclofen or CGP35348 into the NTS of SHR and WKY rats. Data are mean±SE (n=6 rats in each group). *P<0.05 vs. WKY in each group of rats. GABA, γ-aminobutyric acid; GBR, GABAB receptor; HR, heart rate; MAP, mean arterial pressure; NTS, nucleus of the solitary tract.
The effect of the GBR antagonist, CGP-35348, microinjected into the NTS on BP and HR was examined in SHR and WKY rats. Bilateral microinjection of CGP-35348 (100 pmol, 50 nl) into the NTS significantly reduced the MAP in SHR (from 103±7 to 77±5 mmHg, n=6, P<0.01) and WKY rats (from 92±5 to 70±4 mmHg, n=5, P<0.05) (Figure 6C). The depressor response to CGP-35348 was significantly enhanced in SHR rats as compared with WKY rat (Figure 6E). The HR was also reduced by microinjection of CGP-35348 into the NTS in both SHR (from 294±15 to 233±12 beats/min, n=6, P<0.01) and WKY rats (from 289±20 to 257±15 beats/min, n=6, P<0.05) (Figure 6D). The bradycardic response to CGP-35348 was significantly enhanced in SHR as compared with WKY rats (Figure 6F). The data demonstrated that the depressor response to the GBR antagonist was enhanced in SHR as compared with WKY rats.

**Discussion**

The current investigation demonstrated that the BP response to both a GBR agonist and antagonist was elevated in the NTS of SHR, and that GBR expression in the NTS was increased in SHR as compared with WKY rats. This study also provides the first evidence that enhanced endogenous GBR expression in the NTS leads to elevated BP in normotensive rats, indicating that enhanced GBR expression or activity in the NTS may contribute to the development of hypertension in SHR. This conclusion is supported by the following observations: (1) GBR expression was enhanced in the NTS of SHR compared with normotensive WKY rats; (2) GBR1 gene transfer into the NTS significantly increased GBR1 expression and resulted in chronic increases in BP in Wistar rats; and (3) acute microinjection of either exogenous GBR agonist or antagonist into the NTS induced pressor or depressor responses, respectively. These responses were enhanced in SHR, as compared with WKY rats. In contrast, the expression of GABA in the NTS and the responses to the agonist/antagonist microinjected into the NTS were comparable between SHR and WKY rats.

The pressor response to baclofen microinjected into the NTS is elevated in deoxycorticosterone acetate salt-induced hypertensive rats, renal wrap hypertensive rats, and angiotensin II-infusion induced hypertensive rats.6-11 Consistent with these observations, the present study results indicated that acute microinjection of the exogenous GBR agonist (baclofen) into the NTS significantly induced an elevated pressor response in SHR as compared with normotensive WKY rats. The elevated response to GBR agonist could be explained by the current observation that GBR expression was enhanced in the NTS of SHR as compared with WKY rats. Enhanced expression of GBR in the NTS has also been reported in other hypertensive rat models, such as Ang II-induced hypertensive rats, and renal wrap hypertensive rats.6-11 Taken together, the studies demonstrate that enhanced GBR expression in the NTS is associated with hypertension; however, the cellular mechanism(s) underlying enhanced GBR expression in the NTS of SHR is not clear. One possibility is that enhanced GBR expression in the NTS is caused by high BP, because elevated peripheral arterial pressure may increase the central input signal, leading to altered gene expression in brain cardiovascular regulatory areas. This seems unlikely, however, because we have previously observed that GBR expression is unaltered in the NTS of rats made hypertensive by peripheral infusion of L-NNAME.11 A second possibility is that enhanced GBR expression in SHR is caused by angiotensin II, because AngII activity in the NTS is increased in SHR and AngII increases GBR expression.11,14,15

A third possibility is that enhanced GBR expression in the NTS of SHR may be caused by other neural modulators, such as nitric oxide (NO) or reactive oxygen species (ROS). It has been reported that NO production in the NTS of SHR is reduced as compared with WKY rats,16 and enhanced NO production in this brain area by overexpression of endothelial NO synthase lowers BP in SHR.17 On the other hand, ROS generation in the NTS is increased in SHR. Reducing ROS levels either by blockade of ROS-generating enzymes or overexpression of superoxide dismutase will decrease BP in these rats.18,19 However, whether impaired NO production and/or increased oxidative stress in the NTS contributes to enhanced GBR expression in SHR is still unclear. Identifying the exact signaling pathways controlling gene regulation that are altered and lead to elevated GBR expression in the NTS of SHR requires further investigation.

Data from our laboratory, as well as from other research groups, demonstrate that the pressor response to baclofen injected into the NTS is enhanced in the NTS of several hypertensive animal models.3-11 This phenomenon could be explained by several observations showing that GBR expression is elevated in the NTS of these hypertensive rats.9,11 However, it is still unclear whether enhanced GBR expression contributes to the development of hypertension in these animals. Here, we provide the first evidence that viral vector-mediated overexpression of GBR in the NTS results in chronic elevation of BP and HR in normotensive rats, indicating that enhanced GBR expression or activity could contribute to the development of high BP in SHR. However, the exact cellular mechanisms responsible for the elevated BP observed in these rats with overexpressed GBR in the NTS are still unclear. Several possible neuronal pathways could be involved. Anatomic studies showed projections from the NTS to neurons in the PVN of the hypothalamus, a brain area that controls the cardiovascular system via the sympathetic nervous system and hormone release from the pituitary gland. The notion that this pathway is involved is supported by our current observations that microinjection of baclofen into the NTS increased RSNA in both SHR and WKY rats and that the sympathoexcitatory response to baclofen is enhanced in SHR. This hypothesis is also supported by studies from other research groups that show the pressor response to baclofen microinjected into the NTS is attenuated by a vasopressin antagonist.20 In addition, the NTS is well known as a termination site for primary afferent fibers from baroreceptors and other peripheral cardiovascular receptors that contain BP-sensitive neurons. As such, the NTS mediates the inhibitory actions of baroreceptors on sympathetic discharge through neurons in the caudal and rostral ventrolateral medulla (CVLM and RVLM). Therefore, enhanced GABAergic neuronal transmission in the NTS could inhibit baroreceptor afferent input signals triggered by increased BP, leading to dampening of the baroreflex.21 The inhibited baroreflex may contribute to the long-term elevation of BP. This notion is supported by several experimental studies and clinical observations, including: (1) chronically decreasing baroreceptor afferent input to the brain induces sustained hypertension in dogs;22 (2) chronic electrical stimulation of the carotid sinus in dogs produces a sustained decrease in arterial pressure;23 (3) baroreflex input signal reduction induced by sinoaortic denervation causes salt-dependent hypertension in normotensive rats;24 and (4) patients with baroreflex failure suffer from volatile hypertension with increased SNA.25,26 The preponderance of evidence indicates that enhanced GBR-mediated inhibition of the baroreflex may cause central desensitization to elevations in peripheral BP and, thus, reset the BP regulatory set point to a higher
level. This concept is further supported by the observation in the present study that enhanced expression of GBR leads to elevated BP and may contribute to the development of hypertension.

In conclusion, the results of the current study demonstrate that GBR expression is enhanced in the NTS of SHR, as compared with normotensive WKY rats. Moreover, overexpression of GBR in the NTS of normotensive rats results in chronic elevations in BP and HR, suggesting that elevated GBR in the NTS is able to elevate BP. Taken together, the findings suggest that the GABAergic system in the NTS contributes not only to the regulation of BP, but also to central resetting of BP and the development of hypertension.

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Disclosures

None.

References