Sympathoexcitation in Rats With Chronic Heart Failure Depends on Homeobox D10 and MicroRNA-7b Inhibiting GABBR1 Translation in Paraventricular Nucleus

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Background—Chronic heart failure (CHF) increases sympathoexcitation through angiotensin II (ANG II) receptors (AT,R) in the paraventricular nucleus (PVN). Recent publications indicate both γ-aminobutyric acid B-type receptor 1 (GABBR1) and microRNA-7b (miR-7b) are expressed in the PVN. We hypothesized that ANG II regulates sympathoexcitation through homeobox D10 (HoxD10), which regulates miR-7b in other tissues.

Methods and Results—Ligation of the left anterior descendent coronary artery in rats caused CHF and sympathoexcitation. PVN expression of AT,R, HoxD10, and miR-7b was increased, whereas GABBR1 was lower in CHF. Infusion of miR-7b in the PVN caused sympathoexcitation in control animals and enhanced the changes in CHF. Antisense miR-7b infused in PVN normalized GABBR1 expression while attenuating CHF symptoms, including sympathoexcitation. A luciferase reporter assay detected miR-7b binding to the 3′ untranslated region of GABBR1 that was absent after targeted mutagenesis. ANG II induced HoxD10 and miR-7b in NG108 cells, effects blocked by AT,R blocker losartan and by HoxD10 silencing, miR-7b transfection into NG108 cells decreased GABBR1 expression, which was inhibited by miR-7b antisense. In vivo PVN knockdown of AT,R attenuated the symptoms of CHF, whereas HoxD10 overexpression exaggerated them. Finally, in vivo PVN ANG II infusion caused dose-dependent sympathoexcitation that was abrogated by miR-7b antisense and exaggerated by GABBR1 silencing.

Conclusions—There is an ANG II/AT,R/HoxD10/miR-7b/GABBR1 pathway in the PVN that contributes to sympathoexcitation and deterioration of cardiac function in CHF. (Circ Heart Fail. 2016;9:e002261. DOI: 10.1161/CIRCHEARTFAILURE.115.002261.)

Key Words: γ-aminobutyric acid ■ angiotensin II ■ homeobox D10 ■ microRNA-7b ■ paraventricular nucleus

In chronic heart failure (CHF), increased sympathetic signaling exacerbates cardiac stress, both directly and by stimulating fluid retention by the kidneys through increased renal sympathetic nerve activity (RSNA), which is an important cause of morbidity and mortality. The paraventricular nucleus (PVN) participates in the regulation of sympathetic outflow. In CHF, angiotensin II (ANG II) receptor (AT,R) stimulates renal sympathetic nerve activity (RSNA), which leads to fluid retention and venous congestion. ANG II regulates γ-aminobutyric acid signaling in the PVN and excites hypothalamic presympathetic neurons. γ-Aminobutyric acid B receptors (GABBR1 and GABBR2) in the PVN are important for the control of sympathetic activity, but in CHF, their effects are blunted because of GABBR1 gene downregulation. However, the molecular mechanism of decreased GABBR1 gene expression in the PVN during CHF is poorly understood.

See Clinical Perspective

MicroRNAs (miRNAs) contribute to regulate gene expression by posttranscriptional binding to the gene 3′ untranslated region (UTR). miRNA-7b (miR-7b) is highly expressed in the PVN and its expression is upregulated after chronic osmotic stress, indicating a role in fluid and electrolyte...
However, the role of miR-7b and that of its regulation by the transcription factor Homeobox D10 (HoxD10) has not been studied. Furthermore, it has been reported that specific miRNAs play an integral role in the AT_R signaling, especially after activation of the Goq signaling pathway. We hypothesize that during CHF, ANG II stimulates HoxD10, miR-7b, and GABBR1 expression. In cultured HEK293 cells, we determined HoxD10, miR-7b, and an inhibitory antisense oligonucleotide, adenosinovector containing rat GABBR1 siRNA (Ad-siGABBR1) into the PVN, and we measured cardiac function, hemodynamic parameters, anatomic indicators, and sympathetic drive indicators. Furthermore, in vivo, we evaluated AT_R, HoxD10, miR-7b, and GABBR1 expression in the PVN of rats with CHF. In cultured NG108 cells treated with ANG II and the ANG II AT_R blocker losartan (Los), we determined HoxD10, miR-7b, and GABBR1 expression. In cultured HEK293 cells, we determined the binding of miR-7b to the 3′-UTR of GABBR1 gene.

**Methods**

An expanded methods section is available in the Data Supplement.

**Animals**

Male Wistar rats weighing between 180 and 200 g (Changchun Yisi Laboratory Animal Technology Company, Ltd, Changchun, China) were used to induce CHF by coronary ligation as previously described. Catheters were inserted bilaterally into the PVN under anesthesia using stereotaxic technique. Drugs were infused using minipumps at doses based on preliminary experiments and previous studies. Six weeks after coronary ligation, cardiac function was measured using echocardiography under anesthesia providing left ventricular diastolic and systolic diameter, ejection fraction (EF), and fractional shortening (FS). In addition, the rats were anaesthetized and prepared for determination of mean arterial blood pressure (MAP), heart rate (HR), left ventricular end diastolic pressure (LVEDP), left ventricular end systolic pressure, maximum first differentiation of left ventricle pressure (dP/dmin), and RSNA as described previously. After the experiment, tissue was harvested for further analysis.

Four separate groups of animals were used to study the effect of AT_R inhibition with Los on the protein expression of HoxD10 and GABBR1 and quantitative polymerase chain reaction for miR-7b (n=6 for each group): sham-operated control (Sham)+vehicle (Veh), Sham+Los, CHF+Veh, and CHF+Los. Two weeks following coronary artery ligation, 2 subgroups of rats (Los-treated sham and Los-treated CHF) were treated with 10 mg/kg d Los (Merck, Whitehouse Station, NJ) in the drinking water for 4 weeks before killing. All animals received humane care in compliance with Institutional Animal Care and Use Committees of Jilin Normal University, Zhejiang University, Georgetown University, and Uppsa University. Use of animals in this study was confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (the 8th Edition).

**Gene Silencing and Transferring In Vivo**

Recombinant adenoviral vectors (1×10⁹ plaque-forming units/mL) harboring rat AT_R siRNA (Ad-siAT_R, GenBank accession No. NM_74054), rat HoxD10 (Ad-HoxD10, GenBank accession No. NM_00107094), rat GABBR1 siRNA (Ad-siGABBR1, GenBank accession No. NM_031028), or green fluorescent protein siRNA (Ad-siGFP) were manufactured by Vector Gene Technology Company Ltd (Beijing, China). Corresponding virus with null content (Ad-null) or Ad-siGFP was used as a control. To achieve stable knockdown of AT_R or GABBR1 in the PVN, we used adenoviral vectors expressing siRNA targeted against AT_R or control message (GFP), which were constructed and purified. Briefly, 21 base-pair short hairpin RNA targeting sequences specific for AT_R, GABBR1 or GFP were engineered under the control of the rat U6 promoter. The hairpin was situated next to the U6 transcription start site (within 6 base pairs) and followed by a synthetic, minimal polyA cassette. PVN infusions were performed 4 weeks before subsequent procedures to ensure robust transgene expression.

**ANG II Infusion**

After coronary ligation or sham operation, each rat was anesthetized (60 mg/kg ketamine+5 mg/kg xylazine IP) and underwent subcutaneous implantation of osmotic mini pumps (Alzet Model No1004). The pumps were connected to bilateral PVN cannulae for continuous infusion (0.11 µl/h/side) of antagoniR-7b or Ad-siGABBR1 at a total dose of 40 ng/h, NC antagoniR or Ad-siGFP, over a 4-week treatment period. Then the mini pump (Alzet Model No1004) was replaced by another mini pump (Alzet Model No1002). Alzet Model No1002 mini pump were connected to the bilateral PVN cannula for continuous infusion (0.25 µl/h/side) of ANG II at a total dose of 1 ng/kg per min, over a 2-week treatment period.

**Tissue**

Plasma was collected for measuring norepinephrine (NE). Heart tissue was used to measure infarction size as previously described. Brain sections were stored for histology, and PVN samples were isolated using a micropunch technique.

**In Vitro Analysis**

The concentration of NE in the plasma and ANG II in the PVN was quantified using an ELISA kit (Rocky Mountain Diagnostics, Colorado Spring, CO) and performed according to the manufacturer’s instructions. Histological analysis of PVN infusion sites and fluorescence determination of agomiR-7b and antagoniR-7b were performed by standard techniques. Fra-like (Fra-LI) expression was determined by immunohistochemistry to assess neuronal activation in the PVN. GABBR1 immunofluorescence was determined by standard techniques using an anti-GABBR1 antibody (Novus Biologicals, Littleton), Western blot for the transcription factor HoxD10 was performed on nuclear protein extract as previously described. RNA was extracted from PVN micropunches or cultured cells as described in the Data Supplement. Real-time reverse transcriptase polymerase chain reaction quantification of miR-7b level and Western blot analysis for GABBR1 expression were performed by standard techniques (see Data Supplement).

**Synthesis of AgomiRs and miRNA Inhibitor AntagomiRs**

The mature miRNA agomiR-7b and its antisense inhibitor antagoniR-7b were synthesized by GenePharma (Shanghai, China).

**Mutagenesis**

Nucleotide-substitution mutations were performed using polymerase chain reaction–based methods for the 3′-UTRs of GABBR1 gene. For rat genes: MT 3′-UTR of GABBR1 gene: 5′-336-AUUUCAAGCAAGCGAGAGAAAGU-336′ and 5′-633-AGCCUGUCCGCCAAGAGGUG-633′. All constructs were sequence verified. The underlined nucleotides indicate the bases where mutations were made, and the numbers before and after the sequences indicate the positions in GABBR1 gene.

**Cell Culture**

HEK293 cells (American Type Culture Collection, ATCC, Manassas, VA) and NG108 hybrid (neuroblastoma glioma) cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured with...
standard techniques. The ability of miR-7b to reduce GABBR1 expression was studied using a luciferase reporter assay in HEK293 cells. The luciferase assay and transfection procedures in HEK293 cells were performed as described previously.38

NG108 cells were used to study the effect of miR-7b on GABBR1 by using doses of agomiR-7b (50 nmol/L) and antagoniR-7b (100 nmol/L) before Western blot. In separate experiments, Western blot for GABBR1 was done in control cells and cells treated with either NC-agomiR-7b (50 nmol/L), agomiR-7b (50 nmol/L), or antagoniR-7b (50 nmol/L) and antagoniR-7b (100 nmol/L) in combination. Further, NG108 cells were treated with ANG II (100 μM) and Los (1 μM) to study the direct effects on HoxD10 and GABBR1 protein expression with Western blot, as well as real-time polymerase chain reaction quantification of miR-7b.

Finally, the role of HoxD10 in the regulation expression of miR-7b and GABBR1 was studied using siRNA knockdown in NG108 cells after treatment with ANG II (100 μM) and the combination of ANG II (100 μM) and Los (1 μM). siRNA targeting HoxD10 and its negative control were purchased from Santa Cruze Inc (CA, USA).

Data Analysis
Group data are expressed as mean±SEM. Group differences in the data were tested using 2-way analysis of variance followed by comparison for individual group differences using the Tukey’s test or 1-way analysis of variance followed by Dunnett’s test where appropriate. The statistical model for the 2-way analysis of variance used Sham and CHF as one dimension and treatment groups as the other or ANG II and Los as dimensions in those experiments. Statistical significance was indicated by P<0.05. All analyses were performed using GraphPad Prism 5.0.

Results
Development of Heart Failure
Coronary artery ligation caused an average infarct area of 37.4±2.1% (Table I in the Data Supplement), and 90% of infarcts were transmural, whereas no infarcts were identified in Sham. Pleural fluid and ascites were found in CHF but not
in Sham. CHF rats had increased in left ventricular diastolic and systolic diameter (P<0.05) and reduced EF and FS (Table I and II in the Data Supplement). CHF rats had increased LVEDP, the right ventricle (RV)/body weight (BW), and lung/BW ratio, whereas LVSP and +dP/d max were significantly decreased (P<0.05). Heart weight was higher in CHF than in Sham. MAP and HR were not affected by either CHF or Veh infusion into the PVN (Table III in the Data Supplement). CHF rats had higher RSNA and plasma NE (Figure 1A and 1E), which correlated with a higher PVN Fra-LI activity than untreated Sham (Figure 2A and 2B).

MiRNA Treatment In Vivo
Infusion of agomiR-7b into the PVN produced CHF-like changes with increased left ventricular diameters and reduced EF and FS (P<0.05) and exacerbated the changes seen in CHF: In addition, both Sham and CHF had higher LVEDP, RV/BW, and lung/BW ratio (P<0.05), whereas LVSP and +dP/d max were significantly decreased after agomiR-7b compared with artificial cerebrospinal fluid–treated Sham and CHF (P<0.05). The cardiac effects of agomiR-7b were more pronounced in CHF (Table IV and V in the Data Supplement). Further, agomiR-7b increased the sympathetic drive indicators MAP, HR, and RSNA (Figure 1A–1D and 1F), as well as Fra-LI activity (Figure 2A and 2B), in both Sham and CHF. As with the cardiac function and anatomic indicator, changes were more pronounced in CHF.

MiRNA Inhibition In Vivo
AntagomiR-7b attenuated the increase in left ventricle dimension and the deterioration of left ventricular performance as indicated by increased EF and FS (Table II in the Data Supplement) and improved cardiac function by decreasing LVEDP, increasing LVSP, +dP/d max, and –dP/d max in CHF animals as well as in agomiR-7b–treated Sham and CHF (P<0.05; Table IV in the Data Supplement). AntagomiR-7b improved RV/BW and lung/BW ratios in both CHF and agomiR-7b–treated Sham and CHF animals (Table V in the Data Supplement). Neither artificial cerebrospinal fluid nor NC agomiR-7b affected echocardiographic or hemodynamic parameters in CHF or Sham (Table II, IV, and V in the Data Supplement).

AntagomiR-7b was able to reduce MAP, HR, and RSNA in both Sham and CHF. As with agomiR-7b, the responses were stronger in CHF (Figure 1A–1D and 1F). Further, the increase in plasma NE in CHF could be inhibited with PVN antagomiR-7b (Figure 1E), and Fra-LI activity in the PVN could be reduced (Figure 2A and 2B). Subcutaneous administration of antagomiR-7b did not affect sympathetic signaling as indicated by RSNA, plasma NE, or Fra-LI activity in the PVN (data not shown).

Identification of Infusion Sites of Fluorescence-Labeled FAM AgomiR-7b and AntagomiR-7b
Histological analysis showed that agomiR-7b and antagomiR-7b–positive cells were localized in the parvicellular and magnocellular divisions of the PVN (Figure IIA in the Data Supplement). No obvious agomiR-7b and antagomiR-7b–positive cell was found in the adjacent brain regions or other areas throughout the brain in most of the rats (Figure IIB in the Data Supplement).

Molecular Regulation of GABBR1 by miR-7b In Vivo
miR-7b was significantly upregulated in PVN of CHF compared with Sham rats (Figure 3A). miR-7b can bind to the GABBR1 gene 3′-UTR based on computational and bioinformatics analysis using TargetScan (Wellcome Trust Sanger Institute; Figure 3B).

Western blot revealed that downregulation of GABBR1 in PVN of CHF when compared with Sham rats (P<0.05, Figure 3C). There was no change in the supraoptic nucleus (Figure 3C). When the luciferase vector carrying the 3′-UTR of GABBR1 was cotransfected with agomiR-7b or ANG II, respectively, luciferase activity was robustly diminished compared with transfection of a scrambled miRNA or Veh. The inhibitory effect of agomiR-7b was antagonized by its antisense antagomiR-7b. On the other hand, agomiR-7b did not suppress translation of luciferase transcripts containing the mutant GABBR1 gene 3′-UTR (Figure 3D). Further, Western blot showed lower GABBR1 levels in the PVN of CHF rats (Figure 4A) and transfection of agomiR-7b in the NG108 cells, respectively (Figure 4B), and infusion in vivo or transfection in vitro of antagomiR-7b prevented this decrease in vivo (Figure 4A) or in vitro (Figure 4B), respectively. Immunofluorescence showed that CHF rats had fewer GABBR1-positive neurons in the PVN than in the Sham (Figure 4C and 4D). The reduction was exacerbated by agomiR-7b and alleviated by antagomiR-7b. However, GABBR1 mRNA levels were unchanged by agomiR-7b in both CHF and Sham (Figure IIA in the Data Supplement).

Figure 2. Effects of infusion of agomiR-7b and antagomiR-7b into the paraventricular nucleus (PVN) on numbers of Fra-like (Fra-LI)–positive neurons in PVN of Sham and chronic heart failure (CHF) rats. A, Immunohistochemistry for Fra-LI (black dots)–positive neurons in the PVN. Scale bar: 200 μm. B, Bar graph comparing Fra-LI–positive neurons in the PVN. Data are expressed as mean±SEM; n=6 for each group; *P<0.05 vs artificial cerebrospinal fluid (aCSF) or negative control agomiR-7b (NC-agomiR-7b); †P<0.05 vs agomiR-7b; ‡P<0.05 vs antagomiR-7b; ††P<0.05 Sham+aCSF vs CHF+aCSF; 2-way analysis of variance (ANOVA), Tukey test.
Role of PVN ANG II In Vivo

PVN ANG II levels in CHF rats were significantly higher than in Sham rats (P<0.05) (Figure IIIA in the Data Supplement). Infusion of ANG II (0.1, 1, 10 ng/kg per min for 4 weeks) into the PVN increased RSNA and plasma NE (Figure IIIB and IIIC in the Data Supplement). The effect on RSNA and plasma NE was greater at any dose in the CHF+Veh group than in the Sham+Veh group. Infusion of Los prevented this increase. There were no significant differences at any dose between the Sham+Veh and Sham+Los groups (Figure IIIB and IIIC in the Data Supplement).

Knockdown of AT1R in PVN In Vivo

To investigate the cardiovascular consequences of myocardial infarction (MI)–induced AT1R upregulation in PVN, we measured the effects of PVN-targeted Ad-siAT1R on echocardiographic and LV hemodynamic end points in rat 6 weeks after MI or sham surgery. High-resolution ultrasonographic analysis demonstrated normal cardiac function in Sham+Ad-siGFP animals as evidenced by unaltered EF and FS compared with Sham+AT1R rats. Infusion of Los prevented this increase. There were no significant differences at any dose between the Sham+Veh and Sham+Los groups (Figure IIIIB and IIIC in the Data Supplement).

ANG II–Induced Sympathoexcitation Is Rescued by AntagonimR-7b

Rats treated with PVN ANG II+NC antagonimR-7b had increased HR, MAP, RSNA, and plasma NE compared with those of rats treated with PVN NC antagonimR-7b (Table VIII in the Data Supplement). Bilateral PVN infusion of antagonimR-7b prevented the increases in HR, MAP, RSNA, and plasma NE observed in ANG II+NC antagonimR-7b-treated rats (Table VIII in the Data Supplement).

ANG II–Induced Sympathoexcitation Is Enhanced by Silencing of GABBR1

Rats treated with PVN ANG II+Ad-siGFP had increased HR, MAP, RSNA, and plasma NE compared with those of rats...
Effects of In Vivo Knockdown Silencing of AT,R in the PVN on HoxD10, GABBR1 Protein Expression, and miR-7b mRNA Expression

AT,R protein expression in the PVN was increased in CHF+Ad-null rats and reduced by silencing AT,R (Figure...
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IVA in the Data Supplement). HoxD10 protein expression in the PVN was increased in CHF+Ad-null rats and repressed by silencing of AT1R (Figure IVB in the Data Supplement). This was associated with similar changes in miR-7b (Figure IVD in the Data Supplement). However, GABBR1 protein expression in the PVN was decreased in CHF+Ad-null rats and interestingly rescued by silencing of AT1R (Figure IVC in the Data Supplement).

Role of HoxD10 in miR-7b Regulation and GABBR1 Expression In Vivo

HoxD10 protein expression in the PVN was increased in CHF rats and normalized by Los treatment (Figure 5A). This was associated with similar changes in miR-7b (Figure 5C) and a decrease in GABBR1 (Figure 5B). HoxD10 protein expression in the PVN was increased in CHF+Ad-null rats and enhanced by gene transferring of HoxD10 (Figure VA in the Data Supplement). This was associated with similar changes in miR-7b (Figure VC in the Data Supplement) and a decrease in GABBR1 (Figure VB in the Data Supplement).

Molecular Regulation of GABBR1 by miR-7b and HoxD10 In Vitro

Transfection of agomiR-7b reduced the protein expression of GABBR1 in the PVN (Figure 4A) and in cultured NG108 cells (Figure 4B). This effect was abolished by cotransfection of antagomiR-7b (Figure 4A and 4B). AgomiR-7b did not affect GABBR1 mRNA levels in NG108 cells (Figure IIB in the Data Supplement). ANG II (100 µmol/L) increased the expression of HoxD10 protein and miR-7b mRNA expression (Figure 6A and 6C), decreased the expression of GABBR1 protein in NG108 cells (Figure 6B), and pretreatment with Los or silencing of HoxD10 using siRNA (HoxD10 siRNA, siHoxD10) ameliorated the effect in NG108 cells (Figure 6A–6C), but the siHoxD10 negative control oligonucleotides did not (data not shown).

ANG II–Induced Downregulation of GABBR1 Is Rescued by Antagomir-7b

Infusion of ANG II into the PVN downregulated GABBR1 expression (Figure 7). Infusion of antagomir-7b prevented this downregulation. There were no significant differences between NC antagomir-7b and antagomir-7b groups (Figure 7).

Discussion

The main finding in this study was the demonstration of an ANG II/AT1R/HoxD10/miR-7b/GABBR1 signaling pathway, localized to the PVN, regulating sympathoexcitation, and playing a pathogenic role in CHF. Thereby, the present study provides a molecular mechanism for the well-known effect of ANG II on sympathoexcitation in the hypothalamus.

The present result that long-term RSNA is regulated through protein expression effects of miRNA that is stimulated by a specific transcription factor activation by ANG II stimulation of AT1R in the PVN is important because sympathetic nerves directly innervate the distal renal tubule and have a significant influence on sodium balance. Increased renal sympathetic
nerve stimulation is a major mechanism for salt and water retention in patients with circulatory failure. Fluid retention in CHF, although necessary to increase preload and maintain cardiac output, leads to venous congestion and potentially decompensation. Our observations demonstrate that the activity of ANG II and AT1R in the PVN may play a substantial role in the sympathetic stimulation associated with CHF.

We found that CHF increased the expression of HoxD10 protein and miR-7b, whereas the expression of GABBR1 protein was decreased in the PVN. In turn, there were larger effects of infusion of both sense and antisense miR-7b in rats with CHF when compared with Sham. These findings indicate that the ANG II/AT1R/HoxD10/miR-7b/GABBR1 signaling cascade plays an important role in the stimulation of the sympathetic system characteristic of heart failure.

The in vivo findings were reproduced in NG108 cells where an enhanced expression of HoxD10 was found after ANG II treatment, a change that could be inhibited by Los or by transfection with a HoxD10 silencer. These results support our findings of miR-7b inhibition of GABBR1 translation in the PVN, an important mechanism for ANG II–induced sympathectomization, and confirm that this effect is controlled by HoxD10.

Direct AT1R stimulation increased HoxD10 and miR-7b expression in the PVN and decreased GABBR1 expression. AT1R blockade prevented the ANG II–induced changes of HoxD10, miR-7b, and GABBR1. Furthermore, AT1R silencing by using siRNA inhibited the CHF-induced increase of HoxD10, and miR-7b, at the same time, rescued the CHF-induced decrease of GABBR1. This is a novel finding that increases our understanding of the molecular mechanisms driving enhanced sympathetic activity during CHF.

The effect of HoxD10 on the miR-7 promoter and miR-7 expression has been previously reported. The present data
reveals, for the first time, that binding of HoxD10 to the miR-7b promoter is important for the ANG II–mediated miR-7b activation in the central nervous system and the PVN. This finding is supported by our observations that transfection with HoxD10 silencer in ANG II–treated NG108 cells reversed the upregulation of miR-7b nearly back to the control level.

The discovery of HoxD10 and miR-7b expression in the PVN and their regulation by AT1R activation during CHF are new findings of importance in cardiovascular physiology and for the pathophysiology of CHF. Further, the expression of miR-7b in PVN and its important role in the regulation of sympahtoexcitation through the regulation of GABBR1 translation is an important new finding that improves our understanding of how excessive AT1R stimulation may drive long-time effects on sympathetic signaling.

The specificity of the present results was demonstrated using infusions of scrambled miRNA and by peripheral administration of both agonist and antagonist oligonucleotides. Interestingly, the effect of miR-7b on GABBR1 seems to be primarily on the translation level as indicated by changes in GABBR1 protein expression with little effect on mRNA levels, in vivo and in vitro. These observations suggest that after AT1R stimulation and during CHF, the GABBR1 protein is downregulated not by mRNA degradation by miR-7b, but by post-transcriptional regulation. The situation is further complicated by the failure of AT1R silencing in vivo to normalize GABBR1 expression, even though it reduced the activation of HoxD10 and miR-7b.

Sympathetic signaling in CHF is known to be differentiated, with marked increased signaling to the kidneys and the heart, whereas other organs are not as strongly modulated. Accordingly, we have strong indications that cardiac and vascular sympathetic nerve activity is regulated by the ANG II/AT1R/HoxD10/miR-7b/GABBR1 pathway, as measured using HR and MAP, the present data leave unanswered whether the mechanism is important for the differentiation of sympathetic activity between different organs.

In this study, CHF was produced by coronary ligation, which is commonly used model of MI. Cardiac function and compensatory hypertrophy is related to time after ligation and infarct area and were verified in the present project using both cardiac histology, echocardiography, and invasive measurement of cardiac function.

In conclusion, in the PVN, ANG II, by AT1R stimulation, enhances sympathetic activity by activating HoxD10, which increases miR-7b expression and thereby reduces GABBR1 translation. ANG II–induced HoxD10 activation via a miRNA-mediated pathway is a new finding partially clarifying the role of the brain Renin Angiotensin System in cardiovascular regulation. These observations open an interesting area for further study.

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Disclosures
None.

References
16. Li DP, Pan HL. Role of gamma-aminobutyric acid (GABA)A and GABA(B) receptors in paraventricular nucleus in control of sympathetic
Morbidity and mortality associated with chronic heart failure are linked to neurohumoral excitation. Several discrete regions of the central nervous system, such as the paraventricular nucleus, have emerged as primary culprits in driving this neural dysfunction. Although miRNAs within the heart have been implicated in cardiovascular function, it is not known if the function of the miRNA systems in the paraventricular nucleus is altered in the chronic heart failure state. The identification of an miRNA-mediated pathway in the paraventricular nucleus for cardiovascular regulation opens an interesting area for further study. Inhibition and silencing of the angiotensin II/angiotensin II receptors/homeobox D10/microRNA-7b/γ-aminobutyric acid B-type receptor 1 signaling pathway targeted to the hypothalamus may provide a novel strategy for the treatment of chronic heart failure.
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Wang et al: PVN MicroRNA in Sympathoexcitation

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Materials and Methods

CHF model

Male Wistar rats weighing between 180 and 200 grams, were purchased from (Changchun Yisi Laboratory Animal Technology Company, Ltd., Changchun, China) and were used to induce CHF as previously described\(^1\). Briefly, the left thoracotomy was performed through the fifth intercostal space in ventilated isoflurane anaesthetized rats. The pericardium was opened, the heart was exteriorized, and the left anterior descending coronary artery was ligated. Sham-operated rats were prepared in the same manner but did not undergo coronary artery ligation. Approximately 70\% of rats survived coronary artery ligation. CHF rat was identified after 6 weeks of coronary ligation operation. Only the rats with myocardial infarction and an LVEDP above 15 mmHg were considered to be in heart failure\(^2\). All animals received humane care in compliance with Institutional Animal Care and Use Committees of Jilin Normal University, Zhejiang University, Georgetown University and Uppsala University. Use of animals in this study was confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (the 8th Edition, revised 2011).

Synthesis of agomiRs and miRNA inhibitor antagomiRs

The mature miRNA sequence of rno-miR-7b (MIMAT0000780) is 5'-UGGAAGACUUGUGAUUUUGUUGU-3'; rno-agomiR-7b (sense: 5'-UGGAAGACUUGUGAUUUUGUUGU-3'; antisense: 5'-AsAsCAAAAUCACAAGUCUCCsAsUsUs-Chol-3') and its antisense inhibitor
antagomiR-7b were synthesized by GenePharma (Shanghai, China). The sequence of antagomiR-7b is the exact antisense of the mature miRNA sequence (for rat: 5'-AsGsAACAAAUUCACAACGUCUGUCAsCsAs-3'). Additionally, a scrambled and double stranded RNA was used as negative control of agomiR-7b (NC-agomiR-7b), sense: 5'-UUCUCGGACCGUGUCACGUTT-3' and antisense: 5'-AsGsGUGACACGUUCGGGAsTsTs-3'. A scrambled and single-stranded RNA was used as negative control of antagomiR-7b (NC-antagomiR-7b), (for rat: 5'-CsAsGUACUUUGUGUAGAsCsAsAs-3'). The upper case letters represent 2'-OMe-modified nucleotides; subscript ‘s’ represents a phosphorothioate linkage; ‘Chol’ represents cholesterol linked through a hydroxyprolinol linkage.

**Drug infusion into the PVN**

Two weeks after induction surgery the rats were anesthetized, placed in a stereotaxic frame, minipumps were connected to the bilateral PVN, and drugs were infused at doses based on preliminary experiments and previous studies. Two weeks after surgery to establish CHF, the rats were anesthetized (60 mg/kg ketamine+5 mg/kg xylazine, ip) and placed in a stereotaxic frame (Narishige Scientific Instrument Lab, Tokyo, Japan). The dorsal surface of the skull was exposed. A kit connecting osmotic mini-pumps (Alzet Model #1004) to cannulas for continuous infusion (0.11 µl/h/side), was placed within the PVN, bilaterally, through two small holes in the skull (PVN coordinates: 1.8 mm posterior and 0.4 mm lateral to the bregma and 7.8 mm ventral to the dura). Agomir-7b (20 ng/h), antagomir-7b (40 ng/h), or agomir-7b NC (20 ng/h) or antagomir-7b NC (40 ng/h, GenePharma), or artificial
Cerebrospinal fluid (aCSF), were constantly infused over a 4-week treatment period. The total volume of the PVN bilateral and continuous infusion was 200 µl (delivery rate of 0.11 µl/h/side, for 4 weeks). The FAM-labeled constructs were synthesized by GenePharma (Shanghai, China).

**Subcutaneous infusion of drug**

Two weeks after surgery to establish CHF, the rats were anesthetized (60 mg/kg ketamine+5 mg/kg xylazine, ip), separate groups of CHF and Sham rats (six animals each group) were treated with subcutaneous (SC) infusions of a similar dose of the agomir-7b (20 ng/h), antagomir-7b (40 ng/h), or agomir-7b NC (20 ng/h) or antagomir-7b NC (40 ng/h, GenePharma) over a 4-week treatment period. The agomir-7b, antagomir-7b, or agomir-7b NC or antagomir-7b NC was dissolved in artificial cerebrospinal fluid (aCSF; 119 mmol/l NaCl, 3.1 mmol/l KCl, 1.2 mmol/l CaCl₂, 1 mmol/l MgSO₄, 0.50 mmol/l KH₂PO₄, 25 mmol/l NaHCO₃, 5 mmol/l D-glucose, 2.2 mmol/l urea, pH 7.4) for PVN infusion or saline for SC infusion at the desired concentration. Two small incision were made at the base of the neck. Blunt dissection was used to create two small subcutaneous pockets between the scapulae. The two pump (Alzet Osmotic Pump, model#1004, Duret Corporation, Cupertino, CA, USA, 0.11 µl/h/side 4 weeks) were inserted and the skin sutured closed. The method for subcutaneous infusions were based on preliminary experiment and previous studies⁸.

**Knockdown of AT₁R in PVN in vivo**

Four separate groups of animals were used to study the effect of AT₁R knockdown with
Ad-siAT1R on echocardiographic measurements, functional/anatomical indicators and the protein expression of HoxD10 and GABBR1, and qPCR for miR-7b (n=6 for each group): Sham+Ad-siGFP, Sham+Ad-siAT1R, CHF+Ad-siGFP, CHF+Ad-siAT1R. The Ad-siGFP or Ad-siAT1R was infused into bilateral PVN two weeks after the coronary artery ligation or sham operation. The infusion volume was 100 nl for each side of the PVN and the bilateral infusions were completed with a mini-osmotic pump (Alzet, #1004). The final experiment was carried out at the end of the fourth week of the gene transfer.

Recombinant adenoviral vectors (1×10^9 plaque-forming units/ml) harboring rat AT1R siRNA (Ad-siAT1R, GenBank accession No. NM_74054) or green fluorescent protein siRNA (Ad-siGFP) were manufactured by Vector Gene Technology Company Ltd (Beijing, China). Rats were anesthetized with sodium pentobarbital and placed in a stereotaxic apparatus. Viruses were delivered bilaterally to PVN (200 nl total) using titer-matched adenoviral stocks (1×10^9 pfu/ml). Dual virus infusions used titer-matched stocks that were mixed before delivery (100 nl each). The infusion volume was 100 nl for each side of the PVN and the bilateral infusions were completed with a mini-osmotic pump (Alzet, #1004).

HoxD10 gene transfer in vivo

Four separate groups of animals were used to study the effect of HoxD10 gene transfer with Ad-HoxD10 on echocardiographic measurements, functional/anatomical indicators and the protein expression of HoxD10 and GABBR1, and qPCR for miR-7b (n=6 for each group): Sham+Ad-null, Sham+Ad-HoxD10, CHF+Ad-null, CHF+Ad-HoxD10.

Recombinant adenoviral vectors (1×10^{12} plaque-forming units/ml) harboring rat HoxD10
(Ad-HoxD10, GenBank accession No. NM_001107094) were manufactured by Vector Gene Technology Company Ltd (Beijing, China). Corresponding virus with null content (Ad-null) was used as a control. The rats were placed in a stereotaxic frame (Narishige Scientific Instrument Lab, Tokyo, Japan). The stereotaxic coordinates for the PVN were 1.8 mm caudal from bregma, 0.4 mm lateral to the midline and 7.9 mm ventral to the dorsal surface. The Ad-HoxD10 or Ad-null was infused into bilateral PVN two weeks after the coronary artery ligation or sham operation. The infusion volume was 100 nl for each side of the PVN and the bilateral infusions were completed with a mini-osmotic pump (Alzet, #1004). The final experiment was carried out at the end of the fourth week of the gene transfer.

**Echocardiographic measurements**

Four weeks after drug infusion, the changes in left ventricular function were evaluated by transthoracic echocardiography with an ultrasound machine (Vivid 7, GE Medical, USA) equipped with a 10-MHz phased-array transducer. Left ventricular diastolic diameter (LVDd) and left ventricular systolic diameter (LVSD) were measured at the same time, and FS and EF were calculated from M-mode recording as described previously.

**Acute general surgery**

Six weeks after coronary ligation, the rats were prepared for the final experiments under urethane (750 mg/kg ip), α-chloralose (70 mg/kg ip) anesthesia. They received a tracheostomy for mechanical ventilation. A pressure sensor was inserted into the left ventricle via the right carotid artery. Parameters including left ventricular systolic pressure (LVSP), left
ventricular end-diastolic pressure (LVEDP), $+\text{dP/dtmax}$ and $-\text{dP/dtmax}$ were collected$^9, 10$. Then the sensor was retracted to the carotid artery for recording of arterial blood pressure (ABP). Electrocardiogram (ECG) was used to measure heart rate (HR). The left femoral vein was cannulated for drug administration and supplemental doses of anesthesia ($\alpha$-chloralose). Body temperature was maintained at approximately $37 \degree C$.

**Recording of renal sympathetic nerve activity (RSNA) and anatomical measurements**

RSNA was measured as previously described$^{11-13}$ using a BL-420S Data Acquisition & Analysis System (Chengdu TME Technology Co, Ltd, Chengdu, China). In short, left renal artery and vein were exposed via a retroperitoneal approach, and a branch of the renal nerve was isolated and clamped distally to eliminate the afferent activity. The nerve was placed on a bipolar platinum electrode for action potential recording. The signal was expressed as percent of maximum, which was detected after intravenous administration of 10 $\mu g$ sodium nitroprusside (SNP)$^{12}$. Background noise, defined as the signal recorded postmortem, was subtracted from actual RSNA$^{11, 13}$. The right ventricle (RV)/body weight (BW) ratio and lung/BW ratio were measured as described previously$^{5, 14, 15}$.

**Immunohistochemistry for the measurement of indicators of central neural activation**

Fra-like (Fra-LI) expression was determined to assess neuronal activation in the PVN$^{16}$. Rats were anaesthetized with pentobarbital (50mg/kg, ip) and transcardially perfused with PBS and 4% paraformaldehyde. Rats were decapitated while still under deep anaesthesia to collect the brain. Immunohistochemical studies were performed to assess neuronal activation in PVN.
Fra-like (Fra-LI) (fos family gene) expression was used as an indicator of chronic neuronal activation. A general avidin/biotin/peroxidase complex (ABC) procedure was applied\textsuperscript{17}. The neurochemical phenotype of Fra-LI-labeled (Santa Cruz, K-25, sc-253, 1:2000) neurons was determined. Images were captured at 10× magnification using a Diaphot 300 microscope (Nikon, Tokyo, Japan). In each animal, Fra-LI positive neurons within the borders of PVN bilaterally were counted in 2 representative 40-μm transverse sections approximately -1.80 mm from bregma. Manual counts were used to quantify the numbers of Fra-LI positive PVN neurons, and an average value was reported as described previously\textsuperscript{14, 16}.

**Histological analysis and detection of FAM-labelled agomiR-7b and/or antagomiR-7b fluorescence in brain sections**

Histological analysis of PVN infusion sites and fluorescence determination of agomiR-7b and antagomiR-7b were performed by standard techniques\textsuperscript{18}. Rats were anaesthetized deeply with pentobarbital (50 mg/kg, ip) and transcardially perfused with PBS and 4% paraformaldehyde, Brains removed, and 25μm cryosections containing PVN were mounted directly onto glass slides. The location of the pipette tip and infusion into the PVN were confirmed histologically using a Nikon SMZ1500 Stereoscope Fluorescence Microscope (magnification of PVN 4× or magnification of rostral ventrolateral medulla 2×). Rats with micropipette misplacement outside of the PVN according to the rat atlas of Paxinos and Watson\textsuperscript{7} were excluded from group data analysis. If no obvious FAM labelled agomiR-7b and antagomiR-7b-positive cell was found in the PVN including the parvicellular and magnocellular divisions of the PVN, we considered the infusion is missing.
Immunofluorescent Labeling Studies

Brain samples were embedded in optimum cutting temperature medium, and transverse sections were obtained approximately 1.8 mm from bregma. For immunofluorescence, 10 μm sections mounted on slides and stored at -80°C for future use. Sections were air-dried for 60 min at room temperature, washed in PBS for 5 min, and treated in 0.2% Triton X-100 for 15 min. Sections were treated in 50 mM glycine for 15 min, and then incubated with 5% goat serum for 30 min before being washed in PBS three times for 5 min. Sections were incubated with anti-GABBR1 (Novus Biologicals, Littletown, 1:200) for 24 h at 4°C, and then with goat anti-rabbit IgG conjugated FITC secondary antibody (Santa Cruz Biotechnology Inc. USA, 1:200) for 60 min. Sections were studied using a laser confocal microscope Zeiss 710 (Carl Zeiss, Germany) and analyzed by investigators blind to the protocol.

Measurement of myocardial infarction size

Infarction size was determined by standard procedures. After acute general surgery, rats were deeply anaesthesia with pentobarbital (50 mg/kg). The heart was removed, weighed, and fixed in 10% buffered formalin for histological study. The area of infarction was measured as a percentage of the left ventricle using a SigmaScan system (Jandel Scientific, San Rafael, CA) in frozen sections stained with 1% triphenyltetrazolium chloride at 37°C in 0.2 mol/l Tris buffer (pH 7.4) for 30 min.
Micro punch of the PVN

The PVN was isolated by the “punch” technique. At the end of the RSNA recording experiment, all the infused, non-infused, losartan (Los) treated and vehicle (Veh) treated animals were killed with an overdose of pentobarbital. The brain was removed and immediately frozen on dry ice and stored at -70°C until being sectioned. A 450 μm-thick coronal section was cut through the hypothalamus including the PVN, which was punched out with a 15-gauge needle (inner diameter 1.5 mm).

Determination of plasma levels of norepinephrine (NE) and PVN levels of angiotension II (ANG II)

The concentration of NE in the plasma and PVN levels of ANG II was quantified using an ELISA kit (Rocky Mountain Diagnostics, Colorado Spring, Colo), and carried out according to the manufacturer’s instructions.

Effects of infusion of ANG II into the PVN on sympathetic drive indicators in Sham and CHF rats

Two weeks after coronary artery ligation, rats were randomly assigned to the Veh-treated group or the Los-treated group, resulting in four experimental groups: Sham+Veh, Sham+Los, CHF+Veh and CHF+Los. Los-treated rats received 10 mg/kg/day Los in drinking water for period of 4 weeks. Veh-treated rats received normal tap water. ANG II was continuously infused into PVN in three doses (0.1, 1, 10 ng/kg per min for 4 weeks) by an Alzet osmotic minipump.
Effects of infusion of ANG II into the PVN on sympathetic drive indicators in PVN antagomiR-7b treated rats

Rats were randomly assigned to four experimental groups (n=6 for each group): NC antagomiR-7b, NC antagomiR-7b+ANGII, AntagomiR-7b, and ANG II+AntagomiR-7b. NC antagomiR-7b or AntagomiR-7b-treated rats received 40 ng/h, NC antagomiR-7b or AntagomiR-7b in PVN continuous infusion (0.11 μl/h/side) for period of 4 weeks. ANG II was continuously infused into PVN in one dose (1 ng/kg per min for 2 weeks) by an Alzet osmotic minipump.

Effects of infusion of ANG II into the PVN on sympathetic drive indicators in PVN silencing of GABBR1 rats

Recombinant adenoviral vectors (1×10^9 plaque-forming units/ml) harboring rat GABBR1 siRNA (Ad-siGABBR1, GenBank accession No. NM_031028) were manufactured by Vector Gene Technology Company Ltd (Beijing, China).

Rats were randomly assigned to four experimental groups (n=6 for each group): Ad-siGFP, ANG II+Ad-siGFP, Ad-siGABBR1 and ANG II+Ad-siGABBR1. Ad-siGFP or Ad-siGABBR1-treated rats received 40 ng/h Ad-siGFP or Ad-siGABBR1 in PVN continuous infusion (0.11 μl/h/side) for period of 4 weeks. ANG II was continuously infused into PVN in one dose (1 ng/kg per min for 2 weeks) by an Alzet osmotic minipump.
Effect of Los treatment on HoxD10, miR-7b and GABBR1 expression in the PVN in CHF

Two weeks following coronary artery ligation, Los (10mg/kg/day) was given to rats in the drinking water for a period of four weeks. Western blotting for HoxD10 and GABBR1, and qPCR for miR-7b were done in four groups of rats (n=6 for each group): Sham+Veh, Sham+Los, CHF+Veh and CHF+Los.

Cell culture

HEK293 cells (American Type Culture Collection, ATCC, Manassas, VA) and NG108 hybrid (neuroblastoma X glioma) cells (American Type Culture Collection, ATCC, Manassas, VA) were cultured with standard techniques. HEK293 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. NG108 cells (neuroblastoma X glioma) hybrid cells were incubated and grown in high glucose Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G and streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. Cells were seeded in 6-well plates and grown until 60-70% confluent before treatment with agomiR-7b or antagomiR-7b for 24 h.

Luciferase assay

The ability of miR-7b to repress GABBR1 expression was confirmed by luciferase reporter activity assays in HEK293 cells. The luciferase assay was performed as described
previously. In short, the 3'-untranslated region (3'-UTR) of GABBR1 gene was cloned by PCR. Then the constructs were inserted into multiple cloning sites (HindIII and SacI sites) downstream of the luciferase gene in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion, Inc.) as described previously. HEK293 cells (1×10^5/well) were co-transfected with 1 μg pGL3–target DNA (firefly luciferase vector) and 20ng pRL-TK (TK-driven Renilla luciferase expression vector) with ANG II and/or miR-7b mimics (ANG II, ANG II+agomiR-7b, ANG II+antagomiR-7b, NC agomiR-7b, agomiR-7b+antagomiR-7b or antagomiR-7b, respectively). Luciferase activity was measured 48 h after transfection with a dual luciferase reporter assay kit (Promega).

**AgomiR-7b and/or antagomiR-7b treatment on GABBR1 expression in NG108 cells**

NG108 cells were treated with increasing doses of agomiR-7b (50 nmol) and/or antagomiR-7b (100 nmol) before western blotting for GABBR1. In separate experiments western blotting was done in control, NC-agomiR-7b (50 nmol), agomiR-7b (50 nmol), agomiR-7b (50 nmol)+antagomiR-7b (100 nmol) treated NG108 cell lysates.

**Effects of ANG II treatment on HoxD10, miR-7b and GABBR1 expression in NG108 cells**

NG108 cells were treated with ANG II (100 μM) and western blotting will be performed for HoxD10 and GABBR1, real-time PCR will be performed for miR-7b. In a separate experiment western blotting was done in control, ANG II (100 μM) and ANG II (100 μM)+Los (1 μM) treated NG108 cell lysates.
Knockdown of HoxD10 in NG108 cells

In the HoxD10 knockdown experiments NG108 cells were seeded in 6-well plates and grown until 60-70% confluence before treatment with ANG II or Los for 24 h. Transient transfection before ANG II-treatment was performed in NG108 cells using 2.5 μg of plasmid and 10 μl of Lipofectamine 2000 (Invitrogen). The HoxD10-siRNA sequence used in our study is: 5’-CCAGGAGCCCACTAAAGTC-3’ corresponding to rat HoxD10 from 721 to 739 (GenBank No. NM_001107094). siRNA targeting HoxD10 and its negative control were purchased from Santa Cruze Inc. (CA, USA).

Quantitative Real-time PCR for measurement of miR-7b

The PVN and NG108 cells samples total RNA was extracted according to the manufacturer’s instructions using the RNeasy kit (Qiagen, Valencia, CA, USA)\textsuperscript{22}. miR-7b levels were measured using the mirVana qPCR miRNA Detection Kit (Ambion, Texas, USA) in conjunction with real-time qPCR with SYBR Green I. U6 was used as internal control. miRNA was isolated from total RNA using mirVana miRNA Isolation Kit (Ambion). qRT-PCR was performed on a Stratagene Mx3000P thermocycler (Agilent Technologies Inc., Santa Clara, CA, USA) for 40 cycles using specific primers for miR-7b and U6 gene internal control (Table S10). Melting curves were used to separate primer dimer formation from the amplification of miR-7b. Results were expressed as sample gene/reference gene U6 ratio and normalize to Sham, Sham+Veh or NG108 cells Control. Fold variations in expression of miR-7b between RNA samples were calculated.
Quantitative Real-time PCR for measurement of GABBR1 mRNA

GABBR1 mRNA expression was determined by qPCR using standard procedures\textsuperscript{22}. GABBR1 mRNA in the PVN and NG108 cells (American Type Culture Collection, ATCC, Manassas, VA) was measured using real-time qPCR. Total RNA was extracted from PVN and cultured NG108 cells using TRIZOL reagent. Total RNA was subjected to reverse transcription 40min, 38°C in presence of 1.5 μl random primer (Amersham IL) and 400U MMLV reverse transcriptase (USB, Ohio). To detect the level of GABBR1 mRNAs, quantitative Real-time PCR was performed on an ABI 7500 fast Real Time PCR system (Applied Biosystems, USA). The Real-time PCR forward and reverse primer sequences for GABBR1 and β-actin were designed and synthesized (Table S10). β-actin was used as an housekeeping gene. The results were expressed as gene/housekeeping gene ratio and normalized to Control or Sham+NC-agomiR-7b.

Western Blot for the Measurement of GABBR1 and transcription factor HoxD10

Western blotting with nuclear protein extract was performed as previously described\textsuperscript{26, 27}. Total protein fractions from the same samples were used for western blot of GABBR1 after sonication in lysis buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 0.1% Triton X-100, and 1mM PMSF). The homogenate was kept on the ice for 30 min, and centrifuged (20000g, 15min) before the supernatant was collected. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). After boiling the samples for 5 min, the protein samples were fractionated by SDS-PAGE (8%-10% polyacrylamide gels).
and transferred to PVDF membrane (Millipore, Bedford, MA). Milk powder was used for blocking at room temperature for 2 h before incubation with anti-GABBR1 (Novus Biologicals, Littleton, USA, 1:500), anti-HoxD10 (Abcam, HK, 1:500), anti-GAPDH (Santa Cruz Biotechnology Inc. USA, 1:1000) and anti-α-tubulin (Abcam, HK, 1:500) at 4 °C overnight, washed, and incubated with the Alexa Fluor® 700 goat anti-mouse IgG (H+L) or Alexa Fluor® 800 goat anti-rabbit IgG (H+L) (1:4000) for 1 h at room temperature. The results were quantified using Odyssey v1.2 and normalized to GAPDH (as the internal control of GABBR1) or α-tubulin (as the internal control of nuclear transcription factor HoxD10). The final results are expressed as fold changes by normalizing the data to the control values.

References
6. Francis J, MohanKumar SM, MohanKumar PS. Correlations of norepinephrine release in the paraventricular nucleus with plasma corticosterone and leptin after systemic


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sham</th>
<th>CHF</th>
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<tbody>
<tr>
<td>BW, g</td>
<td>411.3±4.3</td>
<td>406.4±6.5</td>
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<tr>
<td>Lung/BW, mg/g</td>
<td>4.6±0.7</td>
<td>10.1±0.8*</td>
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<tr>
<td>RV/BW, mg/g</td>
<td>0.67±0.12</td>
<td>1.08±0.11*</td>
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<tr>
<td>IS, % LV area</td>
<td>0</td>
<td>37.4±2.1*</td>
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<tr>
<td>Minimum thickness of LV, mm</td>
<td>2.4±0.2</td>
<td>0.3±0.04*</td>
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<tr>
<td>LVDd, mm</td>
<td>6.14±0.23</td>
<td>9.31±0.36*</td>
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<tr>
<td>LVSD, mm</td>
<td>3.47±0.16</td>
<td>6.25±0.29*</td>
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<tr>
<td>EF, %</td>
<td>84.2±2.45</td>
<td>60.3±2.09*</td>
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<tr>
<td>FS, %</td>
<td>41.4±1.58</td>
<td>27.6±1.83*</td>
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<tr>
<td>MAP, mmHg</td>
<td>97.9±4.2</td>
<td>91.2±3.6</td>
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<tr>
<td>HR, beats/min</td>
<td>372.5±14.8</td>
<td>397.7±10.9</td>
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<tr>
<td>LVSP, mmHg</td>
<td>141.3±10.9</td>
<td>92.1±13.4*</td>
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<td>LVEDP, mmHg</td>
<td>3.4±1.6</td>
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<td>7265±652</td>
<td>5711±646*</td>
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<td>-dP/dtmax, mmHg/s</td>
<td>6398±536</td>
<td>3974±447*</td>
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BW, body weight; RV, right ventricular weight; IS, infarct size; LV, left ventricle; MAP, mean arterial pressure; HR, heart rate; Sham, sham-operated control; CHF, chronic heart failure; EF, ejection fraction; FS, fractional shortening; LVDd, left ventricle diastolic diameter; LVSD, left ventricle systolic diameter; LVSP, left ventricular systolic pressure; LVEDP, left ventricle end-diastolic pressure; dP/dtmax, maximum first differentiation of left
ventricle pressure. Data are mean±SEM; *P<0.05 vs. Sham groups; one-way ANOVA, Dunnett t-test.
Table S2

**Echocardiographic measurements for the PVN infusion experimental groups (n=6)**

<table>
<thead>
<tr>
<th></th>
<th>LVDd (mm)</th>
<th>LVSd (mm)</th>
<th>EF (%)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>aCSF</td>
<td>5.09±0.24</td>
<td>7.31±0.46</td>
<td>3.54±0.15</td>
<td>5.99±0.14</td>
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<tr>
<td>AgomiR-7b</td>
<td>6.96±0.18*</td>
<td>10.12±0.32*</td>
<td>5.92±0.23*</td>
<td>9.97±0.16*</td>
</tr>
<tr>
<td>AgomiR-7b+AntagomiR-7b</td>
<td>5.23±0.32†</td>
<td>8.01±0.36†</td>
<td>5.03±0.26†</td>
<td>6.17±0.35†</td>
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<tr>
<td>AntagomiR-7b</td>
<td>5.14±0.41</td>
<td>6.18±0.25‡*</td>
<td>3.35±0.34</td>
<td>3.81±0.23‡</td>
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<td>NC agomiR-7b</td>
<td>5.06±0.52</td>
<td>7.47±0.3</td>
<td>3.76±0.42</td>
<td>6.08±0.29</td>
</tr>
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</table>

aCSF, artificial cerebrospinal fluid; Sham, sham-operated control; CHF, chronic heart failure; EF, ejection fraction; FS, fractional shortening; LVDd, left ventricle diastolic diameter; LVSd, left ventricle systolic diameter. Values are mean±SEM; *P<0.05 vs. aCSF or negative control agomiR-7b (NC-agomiR-7b); †P<0.05 vs. agomiR-7b; ‡P<0.05 vs. agomiR-7b+antagomiR-7b; #P<0.05 Sham+aCSF vs. CHF+aCSF; two-way ANOVA, Tukey test.
### Table S3

**MAP and HR for all experimental groups receiving PVN infusion (n=6)**

<table>
<thead>
<tr>
<th></th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>Pre-aCSF: 0 week</td>
<td>98±7</td>
<td>88±3</td>
</tr>
<tr>
<td>Post-aCSF: 1 week</td>
<td>101±4</td>
<td>93±6</td>
</tr>
<tr>
<td>Post-aCSF: 2 week</td>
<td>99±3</td>
<td>96±4</td>
</tr>
<tr>
<td>Post-aCSF: 4 week</td>
<td>104±6</td>
<td>95±5</td>
</tr>
<tr>
<td>agomiR-7b</td>
<td>106±5</td>
<td>91±7</td>
</tr>
<tr>
<td>agomiR-7b+antagomiR-7b</td>
<td>95±9</td>
<td>84±3</td>
</tr>
<tr>
<td>antagomiR-7b</td>
<td>96±3</td>
<td>96±5</td>
</tr>
<tr>
<td>NC agomiR-7b</td>
<td>96±4</td>
<td>84±8</td>
</tr>
</tbody>
</table>

aCSF, artificial cerebrospinal fluid; MAP, mean arterial pressure; HR, heart rate. Values are mean±SEM; two-way ANOVA, Tukey test.
Table S4

Hemodynamic measurements for the PVN infusion experimental groups (n=6)

<table>
<thead>
<tr>
<th></th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+dP/dt max (mmHg/s)</th>
<th>-dP/dt max (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>aCSF</td>
<td>132.2±12.6</td>
<td>87.8±10.4‡</td>
<td>3.7±1.9</td>
<td>15.4±4.3#</td>
</tr>
<tr>
<td>AgomiR-7b</td>
<td>109.5±9.7*</td>
<td>61.8±9.5*</td>
<td>10.1±2.8*</td>
<td>26.5±1.7*</td>
</tr>
<tr>
<td>AgomiR-7b+AntagomiR-7b</td>
<td>139.8±8.5‡</td>
<td>118.5±12.3†</td>
<td>5.3±1.6†</td>
<td>8.3±3.2†</td>
</tr>
<tr>
<td>AntagomiR-7b</td>
<td>145.3±11.4</td>
<td>129.2±10.1‡</td>
<td>3.6±1.5</td>
<td>4.7±2.8‡</td>
</tr>
<tr>
<td>NC agomiR-7b</td>
<td>127.6±12.4</td>
<td>83.7±9.9</td>
<td>3.2±1.2</td>
<td>16.3±5.3</td>
</tr>
</tbody>
</table>

aCSF, artificial cerebrospinal fluid; Sham, sham-operated control; CHF, chronic heart failure; LVSP, left ventricular systolic pressure; LVEDP, left ventricle end-diastolic pressure; dP/dtmax, maximum first differentiation of left ventricle pressure. Values are mean±SEM; *P<0.05 vs. aCSF or negative control agomiR-7b (NC-agomiR-7b); †P<0.05 vs. agomiR-7b; ‡P<0.05 vs. agomiR-7b+antagomiR-7b; #P<0.05 Sham+aCSF vs. CHF+aCSF; two-way ANOVA, Tukey test.
<table>
<thead>
<tr>
<th></th>
<th>RV/BW (mg/g)</th>
<th>Lung/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>CHF</td>
</tr>
<tr>
<td>aCSF</td>
<td>0.67±0.13</td>
<td>1.08±0.14&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>AgomiR-7b</td>
<td>0.86±0.07*</td>
<td>1.33±0.11*</td>
</tr>
<tr>
<td>AgomiR-7b+AntagomiR-7b</td>
<td>0.71±0.09†</td>
<td>0.82±0.10†</td>
</tr>
<tr>
<td>AntagomiR-7b</td>
<td>0.59±0.11</td>
<td>0.74±0.13†</td>
</tr>
<tr>
<td>NC agomiR-7b</td>
<td>0.70±0.12</td>
<td>1.12±0.08</td>
</tr>
</tbody>
</table>

aCSF, artificial cerebrospinal fluid; Sham, sham-operated control; CHF, chronic heart failure; BW, body weight; RV, right ventricular weight.

Values are mean±SEM; *P<0.05 vs. aCSF or negative control agomiR-7b (NC-agomiR-7b); †P<0.05 vs. agomiR-7b; ‡P<0.05 vs. agomiR-7b+antagomiR-7b; ††P<0.05 Sham+aCSF vs. CHF+aCSF; two-way ANOVA, Tukey test.
<table>
<thead>
<tr>
<th>Echocardiographic measurements, functional/anatomical indicators for the PVN knockdown of AT₁R experimental groups (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>lung/BW, mg/g</strong></td>
</tr>
<tr>
<td><strong>RV/BW, mg/g</strong></td>
</tr>
<tr>
<td><strong>IS, % LV area</strong></td>
</tr>
<tr>
<td><strong>LVDd, mm</strong></td>
</tr>
<tr>
<td><strong>LVSD, mm</strong></td>
</tr>
<tr>
<td><strong>EF, %</strong></td>
</tr>
<tr>
<td><strong>FS, %</strong></td>
</tr>
<tr>
<td><strong>LVEDP (mmHg)</strong></td>
</tr>
<tr>
<td><strong>dP/dt (mmHg/s)</strong></td>
</tr>
<tr>
<td><strong>Basal RSNA (% of max)</strong></td>
</tr>
</tbody>
</table>

Sham, sham-operated control; CHF, chronic heart failure; EF, ejection fraction; FS, fractional shortening; LVDd, left ventricle diastolic.
diameter; LVSd, left ventricle systolic diameter. Values are mean±SEM; *P<0.05 vs. Sham+Ad-siGFP; †P<0.05 vs. CHF+Ad-siGFP; two-way ANOVA, Tukey test.
<table>
<thead>
<tr>
<th></th>
<th>Sham+Ad-null</th>
<th>CHF+Ad-null</th>
<th>Sham+Ad-HoxD10</th>
<th>CHF+Ad-HoxD10</th>
</tr>
</thead>
<tbody>
<tr>
<td>lung/BW, mg/g</td>
<td>6.34±0.51</td>
<td>13.12±0.44*</td>
<td>6.47±0.46</td>
<td>17.34±0.62*†</td>
</tr>
<tr>
<td>RV/BW, mg/g</td>
<td>0.68±0.23</td>
<td>1.39±0.36*</td>
<td>0.71±0.34</td>
<td>1.60±0.31*†</td>
</tr>
<tr>
<td>IS, % LV area</td>
<td>0</td>
<td>37±5*</td>
<td>0</td>
<td>39±3*</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>6.35±0.29</td>
<td>8.66±0.57*</td>
<td>6.81±0.35</td>
<td>10.81±0.43*†</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>3.42±0.33</td>
<td>6.26±0.28*</td>
<td>3.84±0.37</td>
<td>8.51±0.42*†</td>
</tr>
<tr>
<td>EF, %</td>
<td>84.2±5.36</td>
<td>49.1±6.62*</td>
<td>80.9±3.74</td>
<td>31.4±4.32*†</td>
</tr>
<tr>
<td>FS, %</td>
<td>41.9±2.83</td>
<td>33.8±1.55*</td>
<td>38.7±2.42</td>
<td>23.2±3.16*†</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4.4±1.2</td>
<td>18.1±3.3*</td>
<td>5.7±1.4</td>
<td>26.8±2.9*†</td>
</tr>
<tr>
<td>dP/dt (mmHg/s)</td>
<td>8514±241</td>
<td>5418±325*</td>
<td>8335±334</td>
<td>4858±223*†</td>
</tr>
<tr>
<td>Basal RSNA (% of max)</td>
<td>19.7±3.1</td>
<td>35.7±5.9*</td>
<td>20.6±5.3</td>
<td>47.2±2.8*†</td>
</tr>
</tbody>
</table>

Sham, sham-operated control; CHF, chronic heart failure; EF, ejection fraction; FS, fractional shortening; LVDd, left ventricle diastolic
diameter; LVSd, left ventricle systolic diameter. Values are mean±SEM; *P<0.05 vs. Sham+Ad-null; †P<0.05 vs. CHF+Ad-null; two-way ANOVA, Tukey test.
### Table S8

**Sympathetic drive indicators for the PVN ANG II and/or antagomiR-7b infusion experimental groups (n=6)**

<table>
<thead>
<tr>
<th></th>
<th>NC antagomiR-7b</th>
<th>ANG II+NC antagomiR-7b</th>
<th>AntagomiR-7b</th>
<th>ANG II+antagomiR-7b</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>376±19</td>
<td>431±22*</td>
<td>368±14</td>
<td>389±16*†</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>91±5</td>
<td>116±4*</td>
<td>88 ±7</td>
<td>101±9*†</td>
</tr>
<tr>
<td>Basal RSNA (% of max)</td>
<td>20.9±4.5</td>
<td>37.3±6.4*</td>
<td>18.2±7.1</td>
<td>24.3±5.6*†</td>
</tr>
<tr>
<td>Plasma NE (pg/ml)</td>
<td>26.4±8.3</td>
<td>48.7±7.6*</td>
<td>24.9±6.4</td>
<td>33.8±8.9*†</td>
</tr>
</tbody>
</table>

HR, heart rate; MAP, mean arterial pressure; RSNA, renal sympathetic nerve activity; Plasma NE, plasma norepinephrine concentration. Data are mean±SEM; *P<0.05 vs. NC antagomiR-7b; †P<0.05 vs. ANG II+NC antagomiR-7b; two-way ANOVA, Tukey test.
<table>
<thead>
<tr>
<th></th>
<th>Ad-siGFP</th>
<th>ANG II+Ad-siGFP</th>
<th>Ad-siGABBR1</th>
<th>ANG II+Ad-siGABBR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>381±23</td>
<td>442±16*</td>
<td>394±15</td>
<td>486±18*†</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>93±8</td>
<td>109±3*</td>
<td>96 ±6</td>
<td>128±7*†</td>
</tr>
<tr>
<td>Basal RSNA (% of max)</td>
<td>18.4±5.1</td>
<td>35.4±7.3*</td>
<td>21.4±6.5</td>
<td>49.2±9.4*†</td>
</tr>
<tr>
<td>Plasma NE (pg/ml)</td>
<td>25.9±7.7</td>
<td>55.3±8.2*</td>
<td>28.1±8.6</td>
<td>69.5±7.9*†</td>
</tr>
</tbody>
</table>

HR, heart rate; MAP, mean arterial pressure; RSNA, renal sympathetic nerve activity; Plasma NE, plasma norepinephrine concentration. Data are mean±SEM; *P<0.05 vs. Ad-siGFP; †P<0.05 vs. ANG II+Ad-siGFP; two-way ANOVA, Tukey test.
Table S10

Reverse transcription specific primers, forward primer and reverse primer sequences for microRNA and GABBR1 mRNA real-time qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse transcription primers 5’ to 3’</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse Primer 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>rno-miR-7b</td>
<td>GTCGTATCCAGTGCGTGGAG</td>
<td>TGGAAGACTTTGATT</td>
<td>CAGTGCCTGTCGTTGAGT</td>
</tr>
<tr>
<td></td>
<td>TCGGCAATTTGCACTGGATACGACAC</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>AACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U6</td>
<td>CGCTTCACGAAATTTGCGTGCAT</td>
<td>GCTTCGGCAGCACATATACAAAT</td>
<td>CGCTTCACGAATTTCGTCGATCA</td>
</tr>
<tr>
<td>GABBR1</td>
<td>Oligo (dT)(_{15}) Primer</td>
<td>GGCTTTAGTCTGGGCTATG</td>
<td>GTCTCAATGGTTCCTGTGC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Oligo (dT)(_{15}) Primer</td>
<td>AGGCCCTCTGAACCCTAAG</td>
<td>TGCCACAGGATCCATACCC</td>
</tr>
</tbody>
</table>
Figure S1

Figure S1. Identification of infusion sites and fluorescence-labelled FAM agomiR-7b and/or antagoniR-7b cells in PVN and adjacent areas. (A) Identification of infusion sites (●) in the PVN, and outside (○). CHF, chronic heart failure; 3V, third ventricle; AHP, anterior hypothalamic area, posterior; f, fornix; LH, lateral hypothalamic area; SM, stria medullaris; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta. (B) Representative section through the PVN of a rat infused bilaterally with FAM labeled agomiR-7b. Scale bar of rostral ventrolateral medulla (RVLM): 500 μm. Scale bar of PVN: 400 μm.
Figure S2. AgomiR-7b had no significant effect on mRNA expression levels of GABBR1 in vivo and in vitro. (A) AgomiR-7b had no significant effect on mRNA expression levels of GABBR1 in the PVN in Sham and CHF rats. Data are expressed as mean±SEM normalized to Sham+NC-agomiR-7b; *P<0.05 vs. Sham+NC-agomiR-7b or Sham+agomiR-7b; two-way ANOVA, Tukey test. (B) AgomiR-7b had no significant effect on mRNA expression levels of GABBR1 in NG108 cells. Data are expressed as mean±SEM normalized to Control; one-way ANOVA, Dunnett t-test.
Figure S3. PVN levels of ANG II in CHF rats and effects of infusion of ANG II into the PVN on sympathetic drive indicators in Sham and CHF rats. (A) Comparison of ANG II level. Data are expressed as mean±SEM; *P<0.05 vs. Sham groups; one-way ANOVA, Dunnett t-test. (B) Summarized RSNA. ANG II (0.1, 1, 10 ng) respectively represents ANG II in three doses (0.1, 1, 10 ng/kg per min for 4 weeks) was continuously infused into PVN by an Alzet osmotic minipump. Data are expressed as mean±SEM; *P<0.05 vs. Sham+Veh; †P<0.05 vs. CHF+Veh; two-way ANOVA, Tukey test. (C) Plasma norepinephrine (NE) concentration. Data are expressed as mean±SEM; *P<0.05 vs. Sham+Veh; †P<0.05 vs. CHF+Veh; two-way ANOVA, Tukey test.
Figure S4. Effects of in vivo knockdown silencing of AT_{1}R in the PVN on expression of
**HoxD10, GABBR1 protein and miR-7b mRNA.** (A) *In vivo* regulation of AT$_1$R protein expression by silencing of AT$_1$R. Right panel, a typical western blot; left panel, data are expressed as mean±SEM normalized to Sham+Ad-siGFP; *P<0.05 vs. Sham+Ad-siGFP; †P<0.05 vs. CHF+Ad-siGFP; two-way ANOVA, Tukey test. (B) *In vivo* regulation of HoxD10 protein expression by silencing of AT$_1$R. Right panel, a typical western blot; left panel, data are expressed as mean±SEM normalized to Sham+Ad-siGFP; *P<0.05 vs. Sham+Ad-siGFP; †P<0.05 vs. CHF+Ad-siGFP; two-way ANOVA, Tukey test. (C) *In vivo* regulation of GABBR1 protein expression by silencing of AT$_1$R. Right panel, a typical western blot; left panel, data are expressed as mean±SEM normalized to Sham+Ad-siGFP; *P<0.05 vs. Sham+Ad-siGFP; †P<0.05 vs. CHF+Ad-siGFP; two-way ANOVA, Tukey test. (D) Silencing of AT$_1$R suppressed miR-7b mRNA expression. Data are expressed as mean±SEM normalized to Sham+Ad-siGFP; *P<0.05 vs. Sham+Ad-siGFP; †P<0.05 vs. CHF+Ad-siGFP; two-way ANOVA, Tukey test.
Figure S5. Effects of in vivo gene transferring of HoxD10 in the PVN on expression of HoxD10, GABBR1 protein and miR-7b mRNA. (A) In vivo regulation of HoxD10 protein expression by overexpression of HoxD10. Right panel, a typical western blot; left panel, data are expressed as mean±SEM normalized to Sham+Ad-null; *P<0.05 vs. Sham+Ad-null; †P<0.05 vs. CHF+Ad-null;
two-way ANOVA, Tukey test. (B) *In vivo* regulation of GABBR1 protein expression by overexpression of HoxD10. Right panel, a typical western blot; left panel, data are expressed as mean±SEM normalized to Sham+Ad-null; *P<0.05 vs. Sham+Ad-null; †P<0.05 vs. CHF+Ad-null; two-way ANOVA, Tukey test. (C) Overexpression of HoxD10 enhanced miR-7b mRNA expression. Data are expressed as mean±SEM normalized to Sham+Ad-null; *P<0.05 vs. Sham+Ad-null; †P<0.05 vs. CHF+Ad-null; two-way ANOVA, Tukey test.