Neuropeptide Y Reverses Chronic Stress-induced Baroreflex Hypersensitivity in Rats

Fang Xie^{1,†}, Lihua Sun^{1,2,†}, Xiaolin Su¹, Ying Wang¹, Jing Liu¹, Rong Zhang^{1,2}, Ning Wang^{1,2}, Jing Zhao¹, Tao Ban^{1,2}, Huifang Niu¹ and Jing Ai^{1,2}

¹Department of Pharmacology, Harbin Medical University (the State-Province Key Laboratory of Biomedicine-Pharmaceutics of China), Harbin, ²Key Laboratory of Cardiovascular Research, Ministry of Education of China, Harbin, [†]Authors with equal contributions to this work

Key Words

Baroreflex sensitivity • Brainstem • CGRP • Chronic stress • GluR2 • GABA_AR • NPY • Substance P

Abstract

Chronic stress, as a risk factor for cardiovascular diseases, has been reported to result in elevated plasma neuropeptide Y (NPY) and be highly associated with abnormal cardiac autonomic function. This study aimed to explore the effect of NPY on the chronic stress-induced abnormal baroreceptor reflex sensitivity (BRS). Seven types of recognized stressors were used to develop chronic stress rat model. Subcutaneously implanting ALZET mini-osmotic pumps containing NPY were used to evaluate the action of NPY on the stressed male rats. We found that chronic stress showed no influence on baseline systolic blood pressure (SBP) and heart rate (HR), whereas NPY (85 µg for 30 days) could elevate baseline SBP and induce bradycardia in rats intervened by various stimuli. NPY pretreatment could preserve chronic stress-induced decreases in left ventricular systolic pressure (LVSP) and the maximum rate of change in left ventricular pressure in the isovolumic contraction period ($+dp/dt_{\max}$) but has shown no effect on left ventricular end diastolic

pressure (LVEDP) and the maximum rate of change in left ventricular pressure in the isovolumic relaxation period ($-dp/dt_{\max}$). Notably, chronic stress led to baroreflex oversensitivity indicated by the elevated ratio of Δ heart rate (HR)/ Δ mean arterial blood pressure (MABP) in rats followed by vasoconstrictor (phenylephrine, PE) or vasodilator (sodium nitroprusside, SNP) administration, which was almost completely reversed by NPY pretreatment. The expressions of substance P (SP) and gamma aminobutyric acid A receptor (GABA_AR) in nucleus tractus solitarius were increased in chronic stress rats, which were counteracted by NPY pretreatment. We conclude that chronic stress-induced baroreflex hypersensitivity could be blocked by NPY pretreatment. Furthermore, the altered expressions of neurotransmitters and receptors in the brainstem might contribute to this process.

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Introduction

Population-based cohort studies suggest that psychosocial factors such as stress and depression are risk factors for cardiovascular diseases (CVD) [1, 2]. The underlying mechanisms linking psychosocial stress

to increased risk for cardiac events may be partially attribute to direct or indirect effects of autonomic dysfunction [3, 4], which is closely associated with heart failure, life-threatening arrhythmias and sudden cardiac death [5]. In addition, numerous studies have reported that various stress led to elevated plasma level as well as tonic expression of neuropeptide Y (NPY), a sympathetic neurotransmitter widely distributed in the cardiovascular neurons of brainstem [6, 7]. Moreover, microinjection of NPY into nucleus tractus solitarius (NTS) induced repression of baroreflex sensitivity (BRS) by glutamate [8]; intravenous administration of NPY caused BRS oversensitivity possibly by influencing on the afferent or central neural connections of baroreflex [9]; and our previous study found that long-term NPY administration in the periphery led to abnormal BRS at least partially by altered glutamate and gamma aminobutyric acid A (GABA) function in NTS [10]. These findings imply the possible role of NPY in the regulation of abnormal BRS induced by stress. Although stress-induced effects of NPY on metabolic syndrome, obesity, vascular remodeling and emotion have been investigated both experimentally and clinically [6, 11, 12], the actions of NPY on the regulation of BRS under stress remain undetermined. Taking into account that NPY is a chronic modulatory peptide characterized by slow onset and persistent action, investigating the stress-induced BRS in animal models provided with lasting NPY administration might be more clinically significant. Unfortunately, the majority of previous stress models were induced by single short-acting stressors, none of which was able to explore the impact of NPY on BRS under long-term integrated stress [13, 14].

In the present study, we first established an integrated stress animal model triggered by a combination of seven recognized stressors [15-18]. The chronic effects of exogenously pretreated NPY on BRS in a 2-week integrated stress rat model were investigated. Moreover, the observed molecular alterations in NTS and nucleus ambiguus (NA) of brainstem might give us some enlightenments about the changes in BRS. Our work might contribute to the further researches into the stress-induced abnormal BRS and benefit the development of therapeutic strategies targeting NPY.

Materials and Methods

Animal

Eighteen adult male Wistar rats (weight 230-250 g) from the Animal Center of the Second Affiliated Hospital of Harbin

Medical University (Harbin, Heilongjiang Province, China) were used in this study. All animal were housed under controlled conditions (humidity: $55 \pm 5\%$; temperature: $23 \pm 1^\circ\text{C}$ and a 12-h light/dark artificial cycle with lights on at 07:00 A.M.) and received food and water *ad libitum*. All studies began at 8:00 A.M.. The animals used in the experimental procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and the regulations of the ethics committees of Harbin Medical University (No. HMUIRB-2008-06). Every care was taken to minimize suffering of all the animals and all efforts were made to minimize the number of animals used.

Treatment Groups

Eighteen rats were equally divided into 3 groups with 6 rats per group as follows: normal control group (Ctl), stimulation group (Sti) and stimulation with NPY ($85 \mu\text{g}$ for 30 days, 3 months) group (Sti + NPY). The rats in Ctl group received neither NPY treatment nor stimulation; the rats in Sti group experienced 2-week integrated chronic stress without NPY administration; and the rats in Sti + NPY group received both 3-month pretreatment of NPY and stress identical to that suffered by the rats in Sti group during the last 2-week NPY treatment.

Implantation of the ALZET Mini-Osmotic Pump

The mini-osmotic pumps (Model 2004, Durect Corporation, Cupertino, CA, USA) were filled with NPY (SciLight Biothechnology, LLC, Beijing, China) (for the rats in Sti + NPY group) or PBS (for the rats in Ctl and Sti group) using a small syringe (1 ml). Each empty pump was weighed together with its flow moderator, and then a syringe was inserted into the pump through its opening at the top after the flow moderator removed. Then the plunger of the syringe was pushed slowly until the solution appeared at the outlet and the flow moderator was fully inserted into the body of the pump. Finally, the filled pumps were weighed again to confirm the volume of fillers occupying over 90% of the mean filling volume. Pumps were incubated in sterile saline at 37°C for 40 h.

All rats were initially anesthetized with intraperitoneal injection (i.p.) of sodium pentobarbital (40 mg/kg). The pumps were surgically implanted subcutaneously in rats as follows: firstly, a small incision was made in the skin between the scapulae on the animals and a small pocket was formed by spreading the subcutaneous connective tissues apart with a hemostat; secondly, the filled pump was inserted into the pocket with the flow moderator pointing away from the incision; finally, the skin incision was closed with wound sutures. The fillers in the pumps could persistently release for a one-month period and the pumps were replaced for new filled ones monthly. All the treatments lasted for 3 months.

Chronic stress management

Rats in the Sti and Sti + NPY groups were subjected to 2-week integrated stress which was similar to that performed by Katz et al. [19]. Briefly, the following stress regimen was sequentially applied: (1) tail suspension, the rat was hung on the hook connected to a metallic gallows (800 mm from the

floor) by an adhesive tape placed 40 mm from the extremity of its tail, and the rats was 150 mm away from the nearest object under acoustical and visual isolation for 6 min [20, 21]; (2) water deprivation, rats were given vacant bottles for 24 h [18]; (3) flashing lights, the rats were exposed to the flashing lights which were 75 watt incandescent bulbs for 10 min [15]; (4) pain stimulation, nip the tail of rats for 1 min [18]; (5) noise treatment, the rats were exposed to an electric bell giving a sound intensity of 90-95 dB SPL at its peak which was in the frequency ranged between 5000-25000 cps lasting 20 sec for 12 times [15]; (6) cold stress, rat was restrained in 3 cm deep cold water ($1 \pm 0.5^\circ\text{C}$) for 5 min [22]; (7) dark treatment, rats were placed in a dark environment for 24 h. All the stressors were administrated in a random sequence for another time throughout the next 7 days and then relative studies were performed in these rats.

Evaluation of body weight, food and drink consumption and biochemical index in blood

The body weight of all rats was recorded at the first and last day of the 14-day stimulation, and then changes of body weight were averaged in each group. Meanwhile, the amount of food and drink consumption for the rats in all groups were recorded per day during the stimulus, and the average for every rat in each group was determined by dividing the total amount of food and drink consumed during the 14 days and the total number of rats in each group. After *in vivo* studies, blood samples taken from the heart were immediately centrifuged (3500 rpm) at 4°C for 10 min to separate the plasma. Then triglyceride (TG), total cholesterol (TC), and low density lipoprotein (LDL-C) in plasma were detected using appropriate kits (Shanghai Rong-sheng Biotech Co., Ltd., China; Cat number 6013E for TC; Cat number 6111D for TG; Cat number 6047H for LDL-C). High density lipoprotein (HDL-C) was also analyzed using commercial kit (Nanjing Jian-cheng Bioengineering Institute, China; Cat number: F003-2).

In vivo cardiac function studies

After 2-week integrated stress, all rats were anesthetized with sodium pentobarbital (40 mg/kg) via i.p. injection. According to previous study [23], cardiac function of all rats was studied. Briefly, arterial blood pressure (ABP) and heart rate (HR) were recorded by inserting the catheter into the left ventricle through the right common carotid artery. To assess the systolic function of heart, left ventricular systolic pressure (LVSP) and the maximum rate of change in left ventricular pressure in the isovolumic contraction period ($+dp/dt_{\max}$) were measured with a BL-420 Data Acquisition & Analysis System (Chengdu Tme Technology Co., Ltd, China). Meanwhile, left ventricular end diastolic pressure (LVEDP) and the maximum rate of change in left ventricular pressure in the isovolumic relaxation period ($-dp/dt_{\max}$) were also studied.

Baroreflex sensitivity study

According to previous study [24, 25], rats were anesthetized initially via i.p. injection of 40 mg/kg sodium pentobarbital. The withdrawal reflex and eye blinking were used to evaluate the anesthetic condition. To maintain the anesthetic state, supplemental doses of anesthetics (0.2 mg/kg sodium

pentobarbital) were administered every 30 min. After the exposure of both the left femoral artery and the right femoral vein, tapered polyethylene catheters with a tip of 0.5 mm diameter were filled with heparinized saline and inserted into the exposed vessels. ABP was monitored in the left femoral artery and the vasoactive drugs were given through right femoral vein.

A blood pressure transducer (model MIT0699, AD Instruments Pty Ltd Castle Hill, NSW Australia) placed in a horizontal position level with the heart was connected to the blood pressure catheter and baseline mean arterial blood pressure (MABP) and HR as well as the MABP and chronotropic responses to the serial vasoactive challenges were recorded. Using the BL-420 Data Acquisition & Analysis System, ABP was measured automatically and HR was evaluated by pulse pressure with the rate-meter function. Vasoconstrictor (phenylephrine, PE) and vasodilator (sodium nitroprusside, SNP) were freshly diluted in 0.9 % NaCl and were injected at different dosages as follows: 16, 32, 64, 128 and 256 $\mu\text{g/ml}$ for PE; 10, 20, 40, 80 and 160 $\mu\text{g/ml}$ for SNP, with a rate of 0.04 ml/100g injected within 3-5 sec. The changes of MABP induced by these dosages of PE and SNP were compared among three groups. Baseline MABP and HR were recorded 30 sec before the application of the first drug. The changes of MABP and relative HR were recorded, and the next challenge was not applied until the ABP and HR reach a plateau. The MABP changes over the baseline ABP level (ΔMABP), the maximal HR responses relative to the baseline HR level (ΔHR) at each dose of PE or SNP administration were averaged by 10 selected points from the elicited trace of blood pressure in order to eliminate statistical error caused by a randomly selected point. To assess the BRS induced by every dose of PE or SNP, the averaged ratio of the HR change over the MABP change ($\Delta\text{HR}/\Delta\text{MABP}$) was calculated. Dose-dependent curves for ΔMABP and $\Delta\text{HR}/\Delta\text{MABP}$ were plotted for all the groups respectively. To demonstrate the maximal HR responses elicited by changed MABP, curves of $\Delta\text{HR}-\Delta\text{MABP}$ were studied. All curves were fitted by Boltzmann equation [26].

Quantitative real-time PCR analysis

To determine whether the mRNA expressions of substance P (SP; SP-sense, 5'-ACA GAT TCC TTT GTT GG-3', and SP-antisense, 5'-GCC TTC TTT CGT AGT TC-3'), calcitonin gene-related peptide (CGRP, CGRP-sense, 5'-CCC TTT CCT GGT TGT CA-3', and CGRP-antisense, 5'-CTC AGC CTC CTG TTC CTC-3'), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2, GluR2-sense, 5'-TGT CCT CCT TTC TCC CT-3', and GluR2-antisense, 5'-CTG AAC CAT CCC TAC CC-3'), gamma aminobutyric acid A receptor (GABA_AR, GABA_AR-sense, 5'-CTG AAG TGA AGA CGG ACA T-3', and GABA_AR-antisense, 5'-ACG CAG GAG TTT ATT GG-3') were altered, 0.5 mg sample of total RNA extracted from brainstem (-600 to +600 μm relative to obex) [27] of rats in each group with Trizol reagent (Invitrogen, Carlsbad, CA) was reverse-transcribed into cDNA using M-MuLV reverse transcriptase and random primers (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative real-time PCR involved a 20 μl reaction mixture prepared with SYBR

Fig. 1. Changes of body weight, food and drink consumption in rats treated with stimulation and NPY. Alterations in (A) body weight, (B) food and (C) drink consumption in rats of control group (Ctl), stimulation group (Sti) and stimulation with NPY group (Sti + NPY). (n = 6, ***P* < 0.01 vs Ctl group; ++*P* < 0.01 vs Sti group).

Groups	TC(mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
Ctl	1.23 ± 0.21	0.56 ± 0.08	0.56 ± 0.13	0.19 ± 0.01
Sti	1.15 ± 0.17	0.48 ± 0.10	0.28±0.07 *	0.26 ± 0.04
Sti + NPY	1.24 ± 0.30	0.55 ± 0.09	0.33 ± 0.02 *	0.25 ± 0.11

Table 1. The effect of chronic stress and Neuropeptide Y (NPY) pretreatment on blood biochemical indexes in rats. Total cholesterol (TC); triglyceride (TG); high density lipoprotein (HDL-C); low density lipoprotein (LDL-C). Ctl: control group; Sti: stimulation group; Sti + NPY: stimulation with NPY group. Mean ± SEM. * *P* < 0.05 vs Ctl group (n=6).

GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) containing an appropriately diluted cDNA solution, 10 nmol/l primer, at 95 °C for 10 min and 40 cycles at 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. Quantitative real-time PCR reactions were analyzed with each sample running twice by using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Warrington, UK). GAPDH (GAPDH-sense, 5'-AAG AAG GTG GTG AAG CAG GC-3', and GAPDH-antisense, 5'-TCC ACC ACC CAG TTG CTG TA-3') mRNA as rat housekeeping gene was measured as an internal control. $\Delta\Delta Ct$ was calculated for every sample, and the expression levels were indicated with $2^{-\Delta\Delta Ct}$.

Western blot analysis

To assess expression levels of proteins in the brainstem (- 600 to + 600 μ m relative to obex), western blot analysis was performed. SP (11 KDa), CGRP (13 KDa), GluR2 (88 KDa) and GABA_AR (70 KDa) were studied. The protein samples were extracted with 600 μ l lysis buffer containing 1% protease inhibitor solution and centrifuged at 12000 g/min for 30 min. The protein content was determined with Sunrise-Basic Tecan (Austria) using bovine serum albumin as the standard. Protein samples were resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The PVDF membrane was incubated with primary antibodies specific for each protein (Santa Cruz, CA, USA) diluted at 1:3000 in PBS buffer (1 h, room temperature). To confirm the specificity of each antibody, the inhibitory peptide was used for each antibody. Samples were incubated with HRP-conjugated secondary antibodies (Santa Cruz, CA, USA) diluted at 1:5000 in the blocking buffer with PBS-T + 5% dry milk (1 h, room temperature) after washing in PBS-T for three times (10 min for each time). Finally, western blot bands were detected using the Odyssey v3.0 software (LI-COR

Bioscience, Lincoln, NE, USA) with the band intensity (area x OD) normalized to β -actin as an index in each group. The expression levels of the detected protein were expressed as relative level by normalizing the data to control values.

Statistical analysis

One-way ANOVA analysis with Spilt-Plot design was performed for beroreflex sensitivity among groups. The significant difference was determined as *P* < 0.05. Comparison between two groups were analyzed by *t* test and data were presented as mean±SEM. Graphpad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was performed to process figures.

Results

NPY preserved chronic stress-induced decrease in body weight but failed to improve the dyslipidemia

In the present study, we found that rats suffering chronic stress displayed significant decrease in body weight (*P* < 0.01 vs Ctl) and food consumption (*P* < 0.01 vs Ctl) but no effect on drink consumption (*P* > 0.05 vs Ctl) compared with age-matched control rats (Fig. 1A, B and C). Three-month NPY pretreatment preserved the decreased body weight in chronic stress rats (*P* < 0.01 vs Sti), while failed to improve the reduced food consumption (*P* > 0.05 vs Sti, Fig. 1A and B). As indicated in Table 1, chronic stress without any effect on TC, TG and LDL-C, resulted in marked decrease in HDL-C level, which was not improved by NPY pretreatment.

NPY altered baseline systolic blood pressure (SBP) and HR in rats with chronic stress

After rats were anesthetized with sodium pentobarbital, the baseline SBP and HR were measured before PE and SNP application. Chronic stress showed no effect on the baseline SBP (Fig. 2A, $P > 0.05$ vs Ctl) and HR (Fig. 2B, $P > 0.05$ vs Ctl) compared with control group. However, When rats were pretreated with 3-month NPY, the baseline SBP level was increased from 114.07 ± 7.56 mmHg in Sti rats to 136.41 ± 8.26 mmHg in Sti + NPY rats (Fig. 2A, $P < 0.05$ vs Sti) and the baseline HR fell to 319.92 ± 15.37 beats/min from 367.2 ± 18.30 beats/min (Fig. 2B, $P < 0.05$ vs Sti).

NPY improved chronic stress-induced cardiac contractile dysfunction

LVSP and $+dp/dt_{\max}$ reflect cardiac contractile function, while LVEDP and $-dp/dt_{\max}$ are indicators of cardiac diastolic function. In this study, we found that rats under chronic stress displayed significantly decreased LVSP. As shown in Fig. 3A, LVSP dropped from 14.28 ± 0.53 kPa in Ctl group to 12.64 ± 0.16 kPa in Sti group ($P < 0.05$ vs Ctl), which was almost completely preserved by NPY pretreatment in Sti + NPY group (14.10 ± 0.56 kPa, $P < 0.05$ vs Sti). In addition, chronic stress obviously drove the $+dp/dt_{\max}$ down from 466.21 ± 30.47 kPa/s in the Ctl rats to 420.15 ± 36.96 kPa/s in the Sti rats (Fig. 3B, $P < 0.05$ vs Sti), and pretreatment with NPY significantly suppressed the stress-induced decrease in $+dp/dt_{\max}$ (Sti + NPY group: 556.51 ± 38.86 kPa/s; $P < 0.05$ vs Ctl, $P < 0.05$ vs Sti). While, there were no significant differences in LVEDP and $-dp/dt_{\max}$ among three groups (Fig. 3C and D, $P > 0.05$).

NPY reversed the baroreflex hypersensitivity during PE application

As shown in Fig. 4A-C, MABP challenged with different doses of PE was gradually increased in three groups. The Δ MABP dose-dependent curve over various PE doses was plotted as Fig. 4D, which demonstrated no obvious difference among the three groups ($F_{0.05(4,56)} = 1.38$, $P = 0.30$). While Δ HR/ Δ MABP ratio against various PE doses, another index to assess BRS, was significantly augmented with the PE doses increase in the Sti rats compared with the Ctl rats ($F_{0.01(4,56)} = 3.16$, $P < 0.001$), which was completely reversed by NPY pretreatment with dosages of 126 μ g/ml and 256 μ g/ml (Fig. 4E, $T = 7.330$ and 8.584 for 128 and 256 μ g/ml, $P < 0.05$). For instance, when 128 μ g/ml of PE was given to rats in all groups, the Δ HR/ Δ MABP grew from 1.57 ± 0.23 beats/

Fig. 2. Baseline systolic blood pressure and heart rate in rats treated with stimulation and NPY. Baseline (A) systolic blood pressure (SBP) and (B) heart rate (HR) in rat of control group (Ctl), stimulation group (Sti) and stimulation with NPY group (Sti + NPY) ($n = 6$; $*P < 0.05$ vs Ctl group; $+P < 0.05$ vs Sti group).

Fig. 3. Quantitative analysis of parameters of cardiac function in rats treated with stimulation and NPY. (A) left ventricular systolic pressure (LVSP); (B) maximum change velocity of left ventricular pressure in the isovolumic contraction period ($+dp/dt_{\max}$); (C) left ventricular diastolic pressure (LVEDP); (D) maximum change velocity of left ventricular pressure in the isovolumic relaxation period ($-dp/dt_{\max}$). Control group (Ctl), stimulation group (Sti) and stimulation with NPY group (Sti + NPY). ($n = 6$; $*P < 0.05$ vs Ctl group, $+P < 0.05$ vs Sti group).

min/mmHg in the Ctl rats to 2.36 ± 0.12 beats/min/mmHg in the Sti rats and fell down to 0.85 ± 0.11 beats/min/mmHg in the Sti + NPY group. Furthermore, the maximal Δ HR to the maximal Δ MABP elicited by increased PE doses were depicted. Fig. 4F indicated that the maximal Δ HR was sharply increased from 63.00 ± 7.59 beats/min in the Ctl rats to 105.59 ± 12.14 beats/min in the Sti rats and fell back to 38.78 ± 6.31 beats/min in Sti + NPY group.

Fig. 4. NPY preserved the baroreflex hypersensitivity induced by chronic stress following phenylephrine (PE) administration. Traces of blood pressure changes induced by PE application at 16, 64 and 128 $\mu\text{g/ml}$ in the rats of (A) control group (Ctl), (B) stimulation group (Sti) and (C) stimulation with NPY group (Sti + NPY). (D) The dose-dependent curve of change in mean arterial blood pressure (ΔMABP) against each dose of PE in the rats of Sti and Sti + NPY group showed no significant difference with that of the Ctl rats challenged by various doses of PE groups ($F_{0.05(4,56)} = 1.38, P = 0.30$). (E) The dose-dependent change of heart rate (ΔHR)/ ΔMABP curve in the Sti rats shifted to the left of the curve of the Ctl rats ($F_{0.01(4,56)} = 3.16, P < 0.001$), and Sti + NPY shifted the curve of the Sti rats to the right with dosages of 128 $\mu\text{g/ml}$ ($T = 7.330, P < 0.05$) and 256 $\mu\text{g/ml}$ ($T = 8.584, P < 0.05$). (F) The maximal ΔHR to the maximal ΔMABP curve of Sti and Sti + NPY rats was plotted by $\Delta\text{HR}/\Delta\text{MABP}$ at each dose. Values are means ($n=6$) with standard errors represented by vertical bars.

Fig. 5. NPY preserved the baroreflex hypersensitivity induced by chronic stress following sodium nitroprusside (SNP) administration. Traces of blood pressure changes induced by SNP application at 10, 40 and 80 $\mu\text{g/ml}$ in the rats of (A) control group (Ctl), (B) stimulation group (Sti) and (C) Sti with NPY group (Sti + NPY). (D) The dose-dependent curve of change in mean arterial blood pressure (ΔMABP) against 10, 20, 40 and 80 $\mu\text{g/ml}$ of SNP in the Sti rats shifted to the left of the curve of the Ctl rats ($F_{0.01(4,56)} = 9.89, P < 0.001$ vs Ctl), and these gains were totally eliminated by the administration of NPY (Fig. 5D, $T = 6.206, 10.166, 8.228$ and $4.152 P < 0.001$ vs Sti). (E) The dose-dependent change in heart rate (ΔHR)/ ΔMABP curve and (F) the maximal ΔHR to the maximal ΔMABP curve of rats for Ctl, Sti, and Sti + NPY group.

NPY reversed the baroreflex hypersensitivity during SNP application

The MABP decrease elicited by injection of SNP showed in a dose-dependent manner in three groups (Fig. 5A-C.). When rats treated with 10 $\mu\text{g/ml}$, 20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$ of SNP, the ΔMABP displayed a compelling disturbed in chronic stress group compared

with Ctl group (Fig. 5D, $F_{0.01(4,56)} = 9.89, P < 0.001$ vs Ctl), and these gains were totally eliminated by the administration of NPY (Fig. 5D, $T = 6.206, 10.166, 8.228$ and $4.152 P < 0.001$ vs Sti). For example, at the dose of 40 $\mu\text{g/ml}$ of SNP, application of NPY preserved the tonic ΔMABP value from $49.05 \pm 7.31 \text{ mmHg}$ in the Sti rats to $22.48 \pm 4.06 \text{ mmHg}$ in the Sti + NPY rats, which was

almost equal to the value in the Ctl rats (18.70 ± 2.80 mmHg). However, at the dose of $160 \mu\text{g/ml}$ of SNP, no significant differences were detected among three groups (Ctl: 57.41 ± 6.19 mmHg; Sti: 56.59 ± 7.22 mmHg; Sti + NPY: 50.00 ± 7.01 mmHg, $P > 0.05$). The $\Delta\text{HR}/\Delta\text{MABP}$ dose-dependent curve of SNP appeared almost flat in the Sti and the Sti + NPY rats, and $\Delta\text{HR}/\Delta\text{MABP}$ value showed a gradual grow following by increased SNP doses (Fig. 5E). Of note, the $\Delta\text{HR}/\Delta\text{MABP}$ values in Sti group at each dose of SNP were all obviously higher than those in Ctl group ($T = 30.799$, $P < 0.001$) and Sti + NPY group (Fig. 5E, $T = 24.960$, $P < 0.05$). This could be fully demonstrated by the ratios induced by $80 \mu\text{g/ml}$ SNP (Ctl: 0.25 ± 0.08 beats/min/mmHg, Sti: 2.54 ± 0.21 beats/min/mmHg, Sti + NPY: 0.60 ± 0.18 beats/min/mmHg). As shown in Fig. 5F, chronic stress led to a prominent increase in the maximal ΔHR to the maximal ΔMABP compared with that in Ctl rats (Ctl: 11.17 ± 4.80 beat/min, Sti: 138.04 ± 12.43 beat/min, $T = 16.307$, $P < 0.001$), which was partially reversed by the administration of NPY (Sti + NPY: 38.73 ± 9.87 beat/min, $T = 12.854$, $P < 0.001$).

The expressions of BRS-related mRNA and protein

SP, CGRP and GluR2 are highly correlated with the glutamatergic transmission, which are essential elements in the baroreflex circuitry. As shown in Fig. 6A and Fig. 7A, the chronic stress strikingly increased the mRNA and protein level of SP by 1.85 ± 0.13 folds ($P < 0.05$ vs Ctl) and 1.67 ± 0.06 folds ($P < 0.05$ vs Ctl) compared with Ctl group, and NPY administration largely diminished the effects of chronic stress on SP at both mRNA (1.28 ± 0.10 folds, $P < 0.05$ vs Sti) and protein (1.22 ± 0.04 folds, $P < 0.05$ vs Sti) level. In accordance with SP, chronic stress showed equal beneficial function on the mRNA (1.47 ± 0.07 folds, $P < 0.05$ vs Ctl) and protein (1.48 ± 0.06 folds, $P < 0.05$ vs Ctl) expression of CGRP. However, NPY failed to influence the actions of the chronic stress on CGRP expression (mRNA: 1.43 ± 0.07 folds, protein: 1.77 ± 0.05 folds, $P > 0.05$ vs Ctl, Fig. 6B and Fig. 7B). As shown in Fig. 6C and Fig. 7C, the expression of GluR2 in both mRNA (0.64 ± 0.07 fold, $P < 0.05$ vs Ctl) and protein (0.72 ± 0.02 fold, $P < 0.05$ vs Ctl) levels were apparently decreased in the Sti rats. Although NPY failed to abolish the reduction at mRNA level (0.62 ± 0.04 fold, $P > 0.05$ vs Sti), the decreased protein expression of GluR2 was indeed soundly preserved by NPY administration (1.29 ± 0.09 folds, $P < 0.05$ vs Sti). Furthermore, by mediating the action of an essential inhibitory transmitter-GABA, GABA_AR also plays a key role in the regulation of BRS. The mRNA and protein

Fig. 6. Baroreflex-related mRNA expression in rats treated with stimulation and NPY. (A) substance P (SP), (B) calcitonin gene-related peptide (CGRP), (C) alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and (D) Gamma-aminobutyric acid A receptor (GABA_AR). Control group (Ctl), stimulation group (Sti) and stimulation with NPY group (Sti + NPY). Values are means of 6 independent experiments, with standard errors represented by vertical bars.

Fig. 7. Baroreflex-related protein expression in rats treated with stimulation and NPY. (A) substance P (SP), (B) calcitonin gene-related peptide (CGRP), (C) alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and (D) Gamma-aminobutyric acid A receptor (GABA_AR). Control group (Ctl), stimulation group (Sti) and stimulation with NPY group (Sti + NPY). Values are means of 6 independent experiments, with standard errors represented by vertical bars.

levels of GABA_AR in Fig. 6D and Fig. 7D were obviously raised to 3.29 ± 0.50 folds ($P < 0.05$ vs Ctl) and 3.20 ± 0.23 folds ($P < 0.05$ vs Ctl), and NPY suppressed both of them to the level paralleled with those in the Ctl rats (mRNA: 1.28 ± 0.11 folds, $P < 0.05$ vs Sti; protein: 1.28 ± 0.03 folds, $P < 0.05$ vs Sti).

Discussion

In the present study, we first report that the BRS was oversensitive in rats under chronic multi-stressor administration, and 3-month pretreatment of NPY could prevent its effect. In addition, the effect of NPY on the stress-induced BRS might partially attribute to the altered expression of SP, GluR2 and GABA_AR.

There were a series of widely used stress animal models induced by a variety of stressors, such as shaker, noise, cold, water deprivation and so on [15–18]. These animal models provided an excellent platform to explore the pathological changes and mechanisms induced by single stressor. To imitate the chronic stress from excessive workload, complex relationships and economic pressure in most people nowadays, the chronic mild stress model [28, 29] similar to ours, was used as a classical model of depression. Compared with those models induced by a single stressor, the integrated stress model in our study resembled the multifactorial intervention chronic stress suffered by people in current society.

Since multiple clinical trials support that autonomic dysfunction contribute to the correlation between depression and increased cardiovascular morbidity and mortality [30–32]. Regardless of the behavioral abnormalities in depression, we focus on the aberrant BRS induced by the similar stimulations. Consequently, this chronic integrated stress model in the present study fits well to our purpose.

As shown in our study, the chronic stress led to obvious decrease in body weight, which was partially preserved by the 3-month pretreatment of NPY. This is consistent with previous report that 3-week different unpredictable mild stressors resulted in a pronounced reduction in body weight which could be repressed by intra-hippocampal injection of 5-HT or NPY [18]. Interestingly, the NPY pretreatment led to a slight rebound of the stress-induced decrease in body weight without any effect on food intake or lipid level. These observations precluded the possibility that central-NPY-induced better appetite ameliorated the chronic stress-induced decrease in body weight [33]. However, the following functions of

NPY might be responsible to its action on the stress-induced decrease in body weight. First, NPY could induce proliferation of endothelial cells and preadipocytes from abdominal fat tissue [12]. Second, NPY originated from sympathetic nerve terminals could suppress not only the lipolytic effects of catecholamines but also the release of leptin [34]. Third, NPY facilitates the maturation of preadipocytes to develop into adipokine-secreting and lipid-storing mature adipocytes [34]. Despite of invariant TC, TG and LDL-C, chronic integrated stress resulted in an evident fall in HDL-C which was implicated in the impairment of cardiovascular system [35]. Regrettably, NPY administration failed to reverse the reduced HDL-C level. Further studies should be performed to elucidate the mechanism behind.

In spite of the exposure to chronic stress, our results showed baseline SBP and HR were constant in the rats of Sti group. This was consistent with previous studies which failed to determine that chronic stress including classical conditioning [36] and operant procedures [37] leads to hypertension. These researchers believed this was not only attributed to the use of normotensive animal model without genetic familial history of hypertension but also the adaptation to the stressors involved [38]. In addition, others reported that only transient rather than lasting elevated BP was induced by psychosocial stress in mice under crowding condition for less than 6 months period, but this observation had not been reproduced in rats stimulated by psychological stress [39]. Collectively, it is reasonable to conclude that the genetic variability, magnitude of stress and duration of stimulation might all participate in the modulation of baseline BP. Moreover, in this study, 3-month pretreatment of NPY in rats under chronic stress resulted in hypertension and bradycardia. According to our previous study conducted in health rats, 4 months of identical NPY administration led to similar changes in baseline SBP and HR as observed in Sti + NPY rats, which might dominantly caused by vasoconstriction-induced pressor action of NPY and its potentiation of other vasoconstrictor agents [40, 41]. Consequently, it is NPY rather than chronic stress played a paramount role in the alterations of baseline SBP and HR in the Sti + NPY rats.

As shown in our results, 2-week chronic integrated stress resulted in BRS oversensitivity when triggered by different doses of PE and SNP injections. This is inconsistent with some other reports, which found impaired BRS after acute mental stress in humans [42, 43] or BRS was constant during psychological stress [44]. As the effects of stress largely depended on the nature

Fig. 8. Schematic diagram illustrating chronic stress and NPY-induced alternations in baroreflex-associated neurotransmitters and receptors in NTS. (A) In physiological condition, gamma-aminobutyric acid (GABA) blocking the release of substance P (SP) in first-order glutamatergic neurons and functioning as an inhibitory transmitter via action on gamma-aminobutyric acid A receptor (GABA_AR) in second-order NTS baroreceptor neurons; the expression and release of SP and calcitonin gene-related peptide (CGRP) facilitate the release of glutamate (Glu) which activates postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and then leads to excitatory conduction of baroreflex afferent neuron within NTS. (B) Chronic stress augments the expression of SP and CGRP, both of which facilitate the release of Glu from glutamatergic afferent terminals. Furthermore, chronic stress leads to elevated GABA_AR expression and a reduced expression of GluR2 in the baroreflex afferent neurons. NPY administration inhibits the increased expression of SP and GABA_AR meanwhile retrieves the attenuated expression of GluR2.

of stressors, it is reasonable to presume that these discrepancies stem from various stress paradigms ranging from a single mental stress to a 2-week chronic integrated stress. Moreover, Farah and colleagues confirmed that acute and chronic stress with the identical stressor-shaker led to opposite BRS-associated cardiac parameters [17]. They found that acute stress resulted in depressed BRS, whereas chronic stress led to an adaptation to the reflex with reduced blood pressure variability (BPV) which represents improved BRS. Notably, the BRS hypersensitivity induced by chronic stress in their findings is in line with the BRS oversensitivity elicited by 2-week integrated stress in the present study.

Interestingly, the chronic stress-induced BRS oversensitivity was almost completely preserved by NPY as demonstrated in the Sti + NPY group, which was constant with our previous study that 4-month lasting NPY administration depressed BRS during PE application. It is interesting to find that when challenged by SNP, 3-moth NPY pretreatment still suppressed the elevated

BRS even though the 4-month NPY administration was confirmed to improve BRS in our previous work. This apparent paradox might emphasize the paramount role of environmental conditions in determining the net effect of certain regulatory factor, namely lasting exogenous NPY could exert opposite effects on health rats and rats suffered from 2-week integrated stress. According to our results, NPY acts as a beneficial factor for the aberrant BRS in this 2-week integrated stress model, which has been further confirmed by the improved parameters of *in vivo* cardiac function (Fig 3). Although baroreflex sensitivity was studied in rats anesthetized with potential BRS-depressive pentobarbital, the alterations in BRS were comparable considering the equal depression effect in all the three groups. Furthermore, in spite of the possible depression of BRS, the stress or NPY-induced changes in BRS in our study were evident enough to confirm the reliability of the present study.

It has been well known that the baroreflex is initiated by a rise in ABP that activates the afferent terminal of

the aortic arch and then transduction to afferent neurons in the nodose ganglia projecting to NTS neurons. Subsequently, cardioinhibitory vagal preganglionic neurons dominantly located in the NA were activated by these neurons in NTS [45]. Considering the prominent roles of NTS and NA in the baroreflex, BRS-relative neurotransmitters and receptors in brainstem were studied to discover the potential molecular mechanism. As glutamate (Glu) and GABA are the prominent excitatory and inhibitory neurotransmitters, abnormal glutamatergic and GABAergic neurotransmissions would disturb the balance of neurotransmitter system. In addition, it has been reported that exogenous NPY administration could penetrate the blood-brain barrier [46], and Y2R could mediate presynaptic GABA release [47]. Furthermore, the associations between Y2R and lines of glutamate-related molecules (metabotropic glutamate receptor 2, three vesicular glutamate transporters and glutamic acid decarboxylase 67) imply the potential role of chronic NPY in regulating glutamatergic neurotransmitter release [48-51]. Glu is an excitory neurotransmitter via action on GluR2 of baroreceptor afferents [52, 53] (Fig.8A). As both SP and CGRP are documented to facilitate the release of Glu [54, 55], we focused on their regulatory effect on glutamatergic function. Our results showed chronic stress significantly increased the mRNA and protein expression of SP and CGRP, which indicated a tonic release of Glu (Fig.8B). In terms of inhibitory neurotransmitter, GABA also plays a key role in baroreflex control of HR by inhibiting parasympathetic outflow [56] and exerts a compelling function in attenuating excessive glutamatergic activity [57]. Ultimately, these chronic stress-induced alterations in neurotransmissions contributed to excessive excitotoxic activity which is detected as BRS oversensitivity in rats with chronic-stress experience. Furthermore, after 2-week integrated stress, the mRNA and protein expression of GluR2 was decreased whereas the expression of GABA_AR showed an opposite change. It is conceivable to presume that the altered expression of GluR2 and GABA_AR are essential protective responses to the excitotoxicity, although they were not pronounced enough to retrieve the homeostasis of excitory and inhibitory neurotransmitters.

Despite of the minor influence in the expression of CGRP, NPY administration significantly suppressed the elevated expression of SP and contributed to the relief of the chronic stress-induced excessive Glu release. In addition, it has been reported exogenously administrated

NPY hampers the synaptic actions of Glu from endogenous sources [8]. In response to the attenuation of the excessive glutamatergic function, the complementary depression of GluR2 expression significantly rebounded and the over-expressed GABA_AR collapsed to the normal level in the Sti and NPY group. Collectively, NPY partially preserved the perturbation of excitory and inhibitory neurotransmitters and benefit the recovery of chronic stress-induced BRS oversensitivity. These molecular findings might facilitate the understanding of the association between NPY and chronic stress-induced BRS oversensitivity.

In summary, this study demonstrated that NPY could preserve the baroreflex oversensitivity induced by chronic stress. In addition, the altered expressions of the BRS-related neurotransmitters and receptors in brainstem partially revealed the mechanism behind. Our results suggest that NPY administration may be a potential protective and therapeutic strategy for chronic stress-induced abnormal BRS as well as the sequential cardiac complications and sudden death.

Abbreviations

ABP (Arterial blood pressure); BRS (Baroreflex sensitivity); CGRP (Calcitonin gene-related peptide); GABA (Gamma aminobutyric acid); Glu (Glutamate); GluR2 (Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2); GABA_AR (Gamma aminobutyric acid A receptor); HDL-C (High density lipoprotein); HR (Heart rate); NA (Nucleus ambiguous); LDL-C (Low density lipoprotein); MABP (Mean arterial blood pressure); NPY (Neuropeptide Y); NTS (Nucleus tractus Solitarii); PE (phenylephrine); SBP (systolic blood pressure); SNP (Nitroprusside); SP (Substance P); TC (Total cholesterol); TG (triglycerides).

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