Long-term Neuropeptide Y Administration in the Periphery Induces Abnormal Baroreflex Sensitivity and Obesity in Rats

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Key Words

NPY • Brainstem • Substance P • GluR2 • GABA_AR

Abstract

Neuropeptide Y (NPY) is an important neuronal element involved in cardiovascular regulation. Since elevated plasma levels of NPY have been observed in numerous pathological situations, this study aimed to determine whether long-term elevated plasma concentrations of NPY could result in aberrant baroreflex sensitivity. Mini-osmotic pump containing NPY (85 µg per 30 days) was subcutaneously implanted between scapulae in male rats for 4 months. The rats treated with NPY showed the following characters compared with control group: (1) attenuated heart rate responding to the increases in mean arterial blood pressure (MABP) induced by phenylephrine, but enhanced heart rate responding to the decreases in MABP induced by sodium nitroprusside; (2) decreased protein levels of substance P (SP) and GluR2, while increased the expression of γ -aminobutyric acid A receptor (GABA_AR) in brainstem; (3) abdominal obesity indicated by increased body weight and accumulated fat mass in

peritoneal cavity; (4) significant increases in total cholesterol, triglycerides, and low density lipoprotein levels in the periphery. These findings indicate that long-term NPY administration in the periphery leads to abnormal baroreflex sensitivity due, at least in part, to the down-regulated expression of SP/GluR2 and elevated expression of GABA_AR in both protein and RNA levels, which indicate the alternations in glutamate function and GABA action in the nucleus tractus solitarius in NPY-treated rats. Furthermore, long-term NPY administration results in abdominal obesity and dyslipidemia.

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Introduction

Impaired baroreflex control of heart rate (HR), as an indicator of abnormal baroreflex sensitivity, is closely related to life-threatening arrhythmias, heart failure and sudden death induced by various diseases, such as cardiac ischemia, diabetes, hypertension, and even depression [1-4]. Within the baroreflex circuitry, both sympathetic and

parasympathetic nervous systems contribute to the regulation of heart function, and the nucleus tractus solitarius (NTS) and nucleus ambiguus (NA) in the brainstem have been considered as the central domain participating in baroreceptor reflex [5].

Neuropeptide Y (NPY) is a sympathetic neurotransmitter present in most of the postganglionic sympathetic nerve terminals in central and peripheral nervous system, and has been implicated in several physiological processes, such as vascular remodeling, bone remodeling, hormone releasing, obesity development, and cardiovascular diseases [6-10]. Substantial clinical data suggest that plasma NPY level is increased in patients with diabetes and hypertension [1, 11-14]. In particular, the level of plasma NPY has been proposed as a biomarker to assess the severity and prognosis of patients suffering from cardiovascular diseases [15]. Among the six identified NPY receptor subtypes, Y1R, Y2R and Y5R are distributed widely in the cardiovascular system [8], with Y1R mediating a vasoconstrictor response, Y2R mediating the decrease of HR and Y5R facilitating cardiac hypertrophy [16-18]. And microinjection of Y1R agonist into NTS led to vasodepressor and bradycardic responses [19]. Moreover, previous studies have demonstrated that central and intravenous injections of NPY immediately affect blood pressure and baroreflex control of HR [20, 21]. Nevertheless, taking into account that NPY is a peptide with slow onset and persistent action, the elevated plasma NPY should exert gradual and long-lasting functions rather than acute and transient effects in chronic pathological situations (i.e. hypertension and diabetes mellitus). Unfortunately, little work has been done to determine the potential molecular mechanism underlying the impact of long-term elevated plasma NPY on baroreflex sensitivity.

In the present study, we found that long-term increased NPY plasma level significantly impaired the baroreflex sensitivity, and the potential molecular mechanism might be imbalanced neuronal function between glutamate and GABA at the level of the brainstem.

Materials and Methods

Animal

Twelve male Wistar rats (weight 230-270 g, aged 3-4 months) were randomly divided into two groups with 6 rats in each group. Each rat in control or NPY group was surgically implanted with an osmotic pump filled with vehicle (PBS) or NPY, respectively. All efforts were made to minimize animal

suffering. The rats were provided by the Animal Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang Province, China) and housed in a room with controlled temperature of $23 \pm 1^\circ\text{C}$ and humidity of $55 \pm 5\%$. Animals were maintained on 12-h dark-light artificial cycle (lights on at 07:00 A.M.) with food and water available *ad libitum*. All studies began at 8:00 A.M.. The use of animals was in accordance with the regulations of the ethics committees of Harbin Medical University (No. HMUIRB-2008-06).

Implantation of the ALZET mini-osmotic pump

Initially, the controlled-release mini-osmotic pumps (Model 2004, Durect Corporation, Cupertino, CA, USA) filled with PBS or 85 μg NPY (SciLight Biotechnology, LLC, Beijing, China) were primed in sterile 0.9% saline solution at 37°C for at least 40 h according to the manufacturer's instructions. Thereafter, the osmotic mini-pumps were implanted subcutaneously in a pocket located between the scapulae on the animals' back (under anesthesia with 40 mg/kg sodium pentobarbital) and were replaced with new pumps containing corresponding filler monthly for 4 months.

Evaluation of plasma NPY-levels, abdominal obesity and dyslipidemia in NPY-treated rats

After the implantation of pumps, body weight, food and drink intake of all rats were monitored daily for 4 months. After 4 months of NPY or PBS treatment, rats in both groups were photographed before and after the exposure of abdominal fat by a digital camera (Fine PixF450, FUJI, Japan). Abdominal fat tissue was stained by hematoxylin and eosin (H&E) and examined under a light microscope (Nikon Elipse 80i, Nikon Corporation, Japan) equipped with Nikon Digital Sight DS-L1 digital camera (Nikon Corporation, Japan). Plasma NPY level was detected using ELISA kit according to the manufacturer's instructions (Cat number EK-049-03, Phoenix Pharmaceutical Company Belmont, CA, USA). Total cholesterol (TC), triglycerides (TG), and low density lipoprotein (LDL-C) were detected using appropriate kits (Cat number 6013E for TC; Cat number 6111D for TG; Cat number 6047H for LDL-C; Shanghai Rong-sheng Biotech Co., Ltd., China), and high density lipoprotein (HDL-C) was measured using kit bought from Nanjing Jian-cheng Bioengineering Institute, China (Cat number F003-2).

Surgical procedure for baroreflex testing

According to previous study [22], rats were anesthetized with sodium pentobarbital (40 mg/kg) via intraperitoneal injection (i.p.). To prevent pedal-withdrawal reflexes and eye blinking, a proper plane of anesthesia was maintained during the entire procedure by supplemental dosing with sodium pentobarbital (0.2 mg/kg) every 30 min. The left femoral artery and right femoral vein were exposed and inserted within tapered tips (0.5 mm in diameter) of two catheters filled with heparinized saline, respectively. Vasoactive drugs were administered via left femoral vein, and blood pressure was recorded through the right femoral artery using a blood pressure transducer (model MIT0699, AD Instruments Pty Ltd Castle Hill, NSW Australia).

Baroreflex sensitivity studies

As described previously [22, 23], the blood pressure catheter was connected to the blood pressure transducer, which was positioned at the level of heart. Using BL-420 Data Acquisition & Analysis System (Chengdu Tme Technology Co., Ltd. China), MABP and HR were recorded automatically. To induce transient increase or decrease in blood pressure, vasoconstrictor (phenylephrine, PE) or vasodilator (sodium nitroprusside, SNP) was injected with different doses (PE: 16, 32, 64, 128, and 256 $\mu\text{g/ml}$; SNP: 20, 30, 40, 80, and 160 $\mu\text{g/ml}$; with a rate of 0.04 ml/100g injected in 3-5 seconds). The next drug administration was performed only when the former MABP and HR responses had reached a plateau (at least 5 min was allowed to elapse between reflex stimulations). The maximal HR response relative to the HR baseline level (ΔHR) and MABP change relative to the MABP baseline level (ΔMABP) induced by each injection of PE or SNP were averaged by 10 selected points from the trace of blood pressure elicited in order to eliminate statistical error caused by a randomly selected point. The ratio of the maximal change in the HR over change in MABP was then calculated and averaged at each dose for each drug in each animal group. Baroreflex sensitivity was estimated by the averaged ratio of the HR change over the MABP change ($\Delta\text{HR}/\Delta\text{MABP}$). As functions of PE and SNP concentration, dose-dependent curves for ΔMABP and $\Delta\text{HR}/\Delta\text{MABP}$ were plotted for each group. Curves of $\Delta\text{HR}-\Delta\text{MABP}$ relationship were also plotted to imply the maximal HR responses induced by MABP changes after the responses reaching a steady plateau. All of the curves were fitted using the Boltzmann equation [22-24].

Quantitative real-time PCR analysis

To evaluate mRNA level of substance P (SP), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and gamma aminobutyric acid A receptor (GABA_AR), total RNA was extracted from rat brainstem (0.5 mg, - 600 to + 600 μm relative to obex) [25] with Trizol reagent (Invitrogen, Carlsbad, CA), and then was reverse-transcribed into single-strand cDNA with M-MuLV reverse transcriptase and random primers (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sequences of gene-specific PCR primers (Shenggong, Shanghai, China) used were as follows: SP-sense, 5'-ACA GAT TCC TTT GTT GG-3' and SP-antisense, 5'-GCC TTC TTT CGT AGT TC-3'; GluR2-sense, 5'-TGT CCT CCT TTC TCC CT-3' and GluR2-antisense, 5'-CTG AAC CAT CCC TAC CC-3'; GABA_AR-sense, 5'-CTG AAG TGA AGA CGG ACA T-3' and GABA_AR-antisense, 5'-ACG CAG GAG TTT ATT GG-3'; GAPDH-sense, 5'-AAG AAG GTG GTG AAG CAG GC-3' and GAPDH-antisense, 5'-TCC ACC ACC CAG TTG CTG TA-3' for control. Quantitative real-time PCR was performed in 20 μl volumes with SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) at 95°C for 10 min and 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, using ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Warrington, UK). GAPDH mRNA was measured as an internal control. $\Delta\Delta\text{Ct}$ was calculated for each sample, and the expression levels were indicated with $2^{-\Delta\Delta\text{Ct}}$.

Western blot analysis

The protein expressions in brainstem (- 600 to + 600 μm relative to obex) tissues were analyzed by Western blot analysis. The proteins under study were: SP (11 KDa), GluR2 (88 KDa), and GABA_AR (70 KDa). Tissues were lysed with 600 μl lysis buffer containing 1% protease inhibitor solution, and then centrifuged at 12000 g/min for 30 min to collect protein extracts in the supernatant. The protein content was determined with Sunrise-basic TECAN (Austria) using bovine serum albumin as the standard. Protein samples were fractionated by SDS-PAGE (10%-20% polyacrylamide gels) and transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Samples were incubated with primary antibodies specific for each protein (Santa Cruz, CA, USA) diluted at 1:3000 in PBS buffer for 1 h at 22°C-23°C. After washing in PBS-T for three times, samples were incubated with HRP-conjugated secondary antibodies diluted at 1:5000 in the blocking buffer with PBS-T and 5% dry milk for 1 h at 22°C-23°C. Western blot bands were quantified using Odyssey v3.0 software (LI-COR Bioscience, Lincoln, NE, USA) by measuring the band intensity (area $\times$ OD) in each group and normalizing to β -actin and the final results were expressed as relative level by normalizing the data to the control values.

Statistical analysis

Data were analyzed by one-way ANOVA. Data between two groups were expressed as mean $\pm$ SEM and compared using Student's t-test. $P < 0.05$ was considered a significant difference. Figures were constructed by GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Results

NPY led to increased baseline systolic blood pressure (SBP) and decreased baseline heart rate (HR)

After 4-month exogenous administration of NPY in the periphery, plasma NPY level was increased to 11.19 ± 0.97 ng/ml from 6.05 ± 0.57 ng/ml in control rats ($n = 6$, $P < 0.05$). Before baroreflex sensitivity study, we measured the baseline SBP (systolic blood pressure) and HR of rats under anesthetic status. Before baroreflex study, baseline SBP was 123.83 ± 4.42 mmHg in NPY rats, which was higher than that in control rats (103.00 ± 6.08 mmHg) ($n = 6$; $P < 0.05$). While the baseline HR in NPY rats was decreased by 16.81% (Ctl: 373.95 ± 6.63 beats/min, $n = 6$; NPY: 310.27 ± 23.66 beats/min, $n = 6$; $P < 0.05$).

NPY reduced the sensitivity of baroreflex control of HR during PE application

As indicated in Fig. 1A and B, MABPs were increased in a dose-dependent manner in response to PE

Fig. 1. Attenuation of baroreflex sensitivity by elevated plasma NPY following phenylephrine (PE) administration. Traces of blood pressure changes induced by PE application at 16, 64 and 128 µg/ml in (A) control rats (Ctl) and (B) NPY-treated rats (NPY). (C) The dose-dependent curve of change in mean arterial blood pressure (Δ MABP) against each dose of PE in rats of NPY group shifted to the right of the curve of Ctl rats (ANOVA; $F_{0.01(4, 40)} = 30.016$, $P < 0.001$). (D) The dose-dependent change of heart rate (Δ HR)/ Δ MABP curve in NPY-treated rats shifted to the right of the curve of Ctl rats (ANOVA; $F_{0.01(4, 40)} = 81.589$, $P < 0.01$). (E) The Δ HR/ Δ MABP curve of rats in control and NPY group was plotted by Δ HR/ Δ MABP at each dose, and the curve of NPY-treated rats shifted to the right of the curve of Ctl rats (ANOVA; $F_{0.01(4, 40)} = 68.329$, $P < 0.01$). Values are means ($n = 6$) with standard errors represented by vertical bars.

Fig. 2. Altered baroreflex sensitivity by elevated plasma NPY during sodium nitroprusside (SNP) application. Traces of blood pressure changes induced by SNP application at 10, 40 and 80 µg/ml in (A) control rats (Ctl) and (B) NPY-treated rats (NPY). (C) The dose-dependent curve of change in mean arterial blood pressure (Δ MABP) against each dose of SNP in rats treated with NPY showed no significant difference with that of Ctl rats with various doses of SNP (ANOVA; $F_{0.05(4, 40)} = 0.324$, $P = 0.585$). (D) The dose-dependent change in heart rate (Δ HR)/ Δ MABP curve of NPY rats shifted to the left of the curve of Ctl rats (ANOVA; $F_{0.05(4, 40)} = 9.277$, $P < 0.05$). (E) The Δ HR/ Δ MABP curve of rats in NPY group was plotted by Δ HR/ Δ MABP at each dose, and the curve of NPY rats shifted to the left of the curve of Ctl rats (ANOVA; $F_{0.05(4, 40)} = 8.023$, $P < 0.05$). Values are means ($n = 6$) with standard errors represented by vertical bars.

in both groups. However, the magnitude of augmented Δ MABP following increased doses of PE injection was

lower in NPY-treated rats than that in control rats (Fig. 1C; ANOVA; $F_{0.01(4, 40)} = 30.016$, $P < 0.001$). For instance,

Fig. 3. Baroreflex-associated mRNA and protein expression in NPY-treated (NPY) and control (Ctl) rats. Relative mRNA levels of (A) substance P (SP), (B) alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and (C) Gamma-aminobutyric acid A receptor (GABA_AR). Relative protein levels of (D) SP, (E) GluR2 and (F) GABA_AR. * $P < 0.05$ vs Ctl. Values are means of six independent experiments, with standard errors represented by vertical bars.

at the dose of 64 $\mu\text{g/ml}$ of PE, the ΔMABP was 24.67 ± 2.83 mmHg in the NPY-treated rats, while it was 37.25 ± 2.31 mmHg in Ctl rats. As an index of baroreflex sensitivity, $\Delta\text{HR}/\Delta\text{MABP}$ ratios were significantly decreased in NPY-treated rats after injection of various doses of PE compared with those in the control rats (Fig. 1D; ANOVA; $F_{0.01(4,40)} = 81.589$, $P < 0.01$). Notably, the hardly changed $\Delta\text{HR}/\Delta\text{MABP}$ values under various doses of PE revealed that the baroreflex sensitivity was almost completely inhibited in NPY-treated rats. In addition, the maximal ΔHR in response to the maximal ΔMABP as another indicator of baroreflex sensitivity was also attenuated in the NPY rats (Fig. 1E; ANOVA; $F_{0.01(4,40)} = 68.329$, $P < 0.01$), which further confirmed the reduction in baroreflex sensitivity along with the elevation in plasma NPY.

NPY increased the sensitivity of baroreflex control of HR during SNP application

As displayed in Fig. 2A and B, MABPs showed a dose-dependent fall over different doses of SNP. However, no significant differences in ΔMABP changes were observed between the two groups even though serial doses of SNP were applied (Fig. 2C; ANOVA; $F_{0.05(4,40)} = 0.324$, $P = 0.585$), which was fully illustrated by the values at the SNP dose of 40 $\mu\text{g/ml}$ (25.70 ± 4.80 mmHg for the Ctl rats and 31.45 ± 3.06 mmHg for the NPY

rats). SNP dose-dependent curves of baroreflex sensitivity ($\Delta\text{HR}/\Delta\text{MABP}$) were plotted and the curves in both groups appeared flat with the curve for the NPY-treated group showing an increased $\Delta\text{HR}/\Delta\text{MABP}$ ratio (Fig. 2D; ANOVA; $F_{0.05(4,40)} = 9.277$, $P < 0.05$). When 80 $\mu\text{g/ml}$ of SNP was given to all rats, NPY treatment resulted in almost doubled $\Delta\text{HR}/\Delta\text{MABP}$ value from 0.22 ± 0.04 beats/min/mmHg to 0.45 ± 0.14 beats/min/mmHg. Although the maximal ΔHR in response to the ΔMABP was increased in NPY-treated rats, the maximal ΔMABP induced by SNP (160 $\mu\text{g/ml}$) was significantly reduced (ΔMABP : 46.23 mmHg) compared with that in the control rats (ΔMABP : 57.41 mmHg) (Fig. 2E; ANOVA; $F_{0.05(4,40)} = 8.023$, $P < 0.05$).

Evaluation of baroreflex-associated mRNA and protein expression

Based on the above findings, we examined whether the long-term elevated plasma NPY influenced certain potential baroreflex-associated mRNAs or protein expression and, at least partially, contributed to the alterations in baroreflex sensitivity. Glutamatergic transmission is a key excitatory regulator in the baroreflex circuitry and is highly related to the expression of SP or GluR2; on the other hand, GABA is a key inhibitory neurotransmitter of central nervous system and acts specifically on GABA_AR in the NTS to participate in cardiovascular

Fig. 4. Long-term NPY administration in periphery leads to abdominal obesity and dyslipidemia. (A) 3-month (NPY/3M) and 4-month NPY (NPY/4M) administration in the periphery induced obese appearance compared with 4-month control rats (Ctl/4M). (B) The body weight of NPY-treated rats (NPY; red) was increased more markedly than that in Ctl rats (Ctl; green). (C) More abdominal fat was accumulated in 4-month NPY-treated rats (NPY/4M) than that in 4-month control rats (Ctl/4M). (D) H&E staining of adipocytes in abdominal fat showed enriched in smaller adipocytes in 4-month NPY-treated rats (NPY/4M) than that in 4-month control rats (Ctl/4M) (original magnification $\times 400$). Drink (E) and Food (F) intake had no significant difference between Ctl rats (green) and NPY rats (red).

Fig. 5. Schematic diagram of altered expression of baroreflex-associated neurotransmitters and receptors induced by elevated plasma NPY. (A) With the physiological level of plasma NPY: gamma-aminobutyric acid (GABA) braking the release of substance P (SP) in first-order glutamatergic neurons and functioning as an inhibitory transmitter in second-order neurons of baroreflex afferent neuron within nucleus tractus solitarius (NTS) via action on gamma-aminobutyric acid A receptor (GABA_AR); the pairing release of glutamate (Glu) and SP activating postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subunit 2 (GluR2) and then leading to excitatory conduction of baroreflex afferent neuron within NTS. (B) With elevated level of plasma NPY: the expression of GABA_AR increasing in second-order neurons of baroreflex afferent within NTS; meanwhile the release of Glu being reduced due to the decreased expression of SP and GluR2 and finally leading to faintish excitatory conduction.

regulation. Thus, we detected the alterations of these baroreflex-associated proteins in the brainstem at both

mRNA and protein levels. The mRNA and protein levels of SP were dramatically decreased by 0.63 ± 0.07 and

Groups	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
Ctl	1.24 ± 0.08	0.56 ± 0.03	0.56 ± 0.05	0.17 ± 0.05
NPY	1.60 ± 0.16*	0.92 ± 0.16*	0.37 ± 0.07*	0.59 ± 0.10*

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

0.52 ± 0.06 fold, respectively (Fig. 3A and D; $P < 0.05$), the mRNA and protein levels of GluR2 were reduced by 0.82 ± 0.10 (Fig. 3B; $P > 0.05$) and 0.55 ± 0.04 fold (Fig. 3E; $P < 0.05$), respectively, in the NPY group compared with those in the control group. As for the expression of GABA_AR, the mRNA and protein expression levels were 2.30 ± 0.32 and 1.58 ± 0.06 folds greater in the NPY rats than those in the control rats (Fig. 3C and F; $P < 0.05$).

Long-term NPY administration in the periphery leads to abdominal obesity and dyslipidemia

In accordance with previous report [26], exogenous application of NPY in the periphery induced significant obesity compared with age-matched control rats (Fig. 4A). The obesity phenotype was verified by marked increase in body weight and accumulated fat mass in peritoneal cavity (Fig. 4B and C). H&E staining of adipocytes in abdominal fat showed enriched in smaller adipocytes in NPY rats than that in control rats (Fig. 4D). However, no differences in food and drink consumption were observed between the control rats and the NPY rats (Fig. 4E and F).

It has been known that obesity is closely associated with dyslipidemia. We therefore detected biochemical indexes related to lipid metabolism. Four-month treatment of NPY in the periphery elicited hyperlipidemia. NPY significantly increased TC, TG as well as LDL-C, and decreased HDL-C in blood (Table 1).

Discussion

In the present study, by applying exogenous NPY in a slow-release mini-osmotic pump delivery system for 4 months, we found that chronic NPY treatment resulted in abdominal obesity and hyperlipidemia in rats. Most importantly, we provided the first evidence that long-term elevated plasma NPY impaired the baroreflex sensitivity, which might be attributed, at least in part, to the reduction

in SP and GluR2 expression, and elevation in GABA_AR expression in brainstem.

Since high circulating NPY was found in patients suffering from diabetes, hypertension as well as cardiac disease [8, 11, 13, 14], and baroreflex sensitivity was severely impaired in patients with diabetes or hypertension [1, 27, 28], we speculate that NPY may participate in this process. Though previous studies have reported that central and intravenous injections of NPY led to changes in the ABP and HR [20, 29], the chronic effect of NPY on baroreflex sensitivity has not yet been clarified under disease status. Interestingly, in the present study, we found that the baseline SBP was significantly increased in 4-month NPY-treated rats, which was inconsistent with the findings from previous research that transient injection of NPY into NTS led to an initial and reversible fall in the SBP and HR [20]. The disparate effects of NPY might be substantially due to the following three considerations. Firstly, these studies differed in the administration methods: cranial and subcutaneous administrations might dominantly exert central and systematic functions, respectively. Secondly, the durations of NPY administration were unequal, which ranged from a single-dose injection to a 4-month treatment. Thirdly, C- and N-terminal of NPY show exactly opposite effect on BP and HR [19, 30].

To further explore the effect of long-term elevated plasma NPY on cardiac autonomic function, baroreflex control of HR was studied. Our observation showed that 4-month systemic NPY administration drove both the PE and SNP dose-dependent curves for $\Delta\text{HR}/\Delta\text{MABP}$ to be flat (Fig. 1D, Fig. 2D) suggesting the dramatic impairment of baroreflex sensitivity. Furthermore, NPY substantially inhibited the reflex bradycardia caused by PE-induced acute elevation of MABP (Fig. 1E). Interestingly, NPY treatment facilitated the reflex tachycardia stimulated by SNP-induced transient depression of MABP (Fig. 2E). The observed disparity favors the previous hypotheses that two populations of

neurons or pathways might exist in the brain: one mediates depressor responses during increase in blood pressure, whereas the other underlies pressor responses during decrease in blood pressure [23].

In order to further confirm the action of long-term NPY administration on the baroreflex sensitivity and exclude the negative effects of anesthetics on it, we performed qRT-PCR and Western blot to assess baroreflex-associated molecular changes. According to recent report, cell body expressed Y2R also co-expressed GABA, which is mostly presynaptic since Y2R nerve terminals appeared around GABA-immunoreactive cells, suggesting Y2R have a modulatory role in mediating presynaptic GABA release [31]. Meanwhile, many other transmitter- and signalling- related molecules are considered to be associated with the Y2R, including glutamic acid decarboxylase 67, the three vesicular glutamate transporters, and the metabotropic glutamate receptor 2, providing evidence for studying the regulating effect of chronic NPY on glutamatergic neurotransmitter release [32-35]. In addition, glutamate (Glu) and GABA are principal excitatory and inhibitory neurotransmitters which play a critical role in the control of HR as well as cardiac function [36-39]. NPY has also been reported to penetrate the blood-brain barrier by exogenous NPY application [40]. So, we have a reason to believe that NPY may play a central and functional role with positive or negative influence on the regulator proteins in both glutamate and GABA pathway at either protein or mRNA levels in the brainstem. Therefore, we decided to examine the changes of Glu and GABA-related proteins. It is well known that the molecular mechanism of baroreflex control of HR in NA is mediated by glutamatergic transmission [41]. Electrical stimulation of NTS evokes fast and slow postsynaptic potentials in the NA cardiac neurons that are mediated by both non-NMDA (AMPA or kainate) and NMDA receptors [39]. In particular, SP has been reported to enhance the release of glutamate and hence modulate glutamatergic transmission [42]. Consequently, the action of glutamate on GluR2 could decrease the blood pressure and endogenous SP could enhance the baroreflex sensitivity in rats [43-45]. Moreover, it has been demonstrated that exogenous NPY injection into the NTS of rats suppresses the synaptic actions of endogenously released Glu [20]. We therefore detected the expression of SP and GluR2 in NTS in order to observe whether long-lasting elevated plasma NPY could affect the glutamatergic pathway resulting in the altered baroreflex sensitivity. In accordance with our hypothesis, the repressed mRNA and protein expressions of SP and GluR2

in NTS from NPY-treated rats (Fig. 3A, B, D, E) suggested that elevated plasma NPY might indirectly antagonize the actions of glutamatergic transmission and finally led to increased baseline MABP and attenuated baroreceptor reflexes (Fig. 5B). These results further confirmed that microinjection of NPY into the NTS inhibited Glu responsiveness and attenuated baroreflex sensitivity [20], which might be due to the direct action of NPY on Y1R in NTS [19]. These findings suggest that the mechanisms of long-term NPY may differ from that of acute NPY administration. Moreover, the increased BP in NPY-treated rats in the present study is inconsistent with the decreased BP induced by microinjection of NPY in the NTS. This disparity may also be attributed to that long-term NPY exerts its function on BP or HR by indirect mechanism rather than direct action on Y1R or Y2R. In addition, GABA could decrease parasympathetic outflow via action on GABA_AR in the second-order NTS neurons and then contribute to increased SBP and impaired baroreflex sensitivity [46]. Furthermore, GABA could inhibit Glu via the action on predominating heterosynaptic GABA_B receptors and then influence excitatory and inhibitory balance (Fig. 5A) [47]. Augmented expression of GABA_AR elicited by elevated plasma NPY in the present study (Fig. 3 C and F) suggested that the raised SBP and aberrant baroreflex sensitivity might at least partially be attributed to tonic GABAergic function (Fig. 5B), which might also rely on the modulatory effect of NPY by acting on Y2R in mediating presynaptic GABA release [31]. Undoubtedly, it is an interesting topic to identify the exact mechanisms by which elevated plasma NPY altered the baroreflex sensitivity.

In accordance with previous report [26], exogenous NPY administration in the periphery induced obvious abdominal obesity and enrichment of smaller adipocytes (Fig. 4C, D). Interestingly, the body weight of NPY-treated rats increased dramatically without any changes in drink or food consumption (Fig. 4 B, E, F), suggesting that NPY could also induce obesity by non-central pathway, which might influence adipocyte differentiation rather than appetite. Since the typical abdominal obesity induced by NPY is an independent risk factor of significant dyslipidemia and type 2 diabetes [48, 49], we presume that increased NPY in diabetic patients may participate in the process of abdominal obesity and dyslipidemia.

Based on above results, we provided new information about the effects of NPY on the baroreflex circuitry. These findings conclude that long-term NPY

administration in the periphery could lead to abdominal obesity and dyslipidemia, which is independent to central mechanism. Furthermore, the abnormal baroreflex sensitivity in NPY-treated rats may be explained by attenuated glutamate function and elevated GABA action in the NTS.

Abbreviations

ABP (Arterial blood pressure); BRS (Baroreflex sensitivity); 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 ambiguus); LDL-C (Low density lipoprotein);

MABP (Mean arterial blood pressure); NPY (Neuropeptide Y); NTS (Nucleus tractus solitarius); PE (Phenylephrine); SBP (Systolic blood pressure); SNP (Sodium nitroprusside); SP (Substance P); TC (Total cholesterol); TG (Triglycerides).

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