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Original Article

Apelin in the hypothalamic paraventricular nucleus improves cardiac function in surgical trauma rats

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Abstract: Apelin is a novel endogenous active peptide. The aim of this study is to investigate whether apelin in the paraventricular nucleus (PVN) can improve the cardiac function in rats subjected to thoracic surgery trauma, and whether it is involved in the protective effect of electro-acupuncture (EA). Sprague-Dawley rats were randomly divided into non-stressed group (control), thoracic surgical trauma stressed group (trauma) and bilateral Neiguan EA applied on thoracic surgical trauma stressed group (trauma + EA-PC 6). The mRNA expressions of apelin receptor (APJR) and apelin in the PVN were detected by real time-PCR. The exogenous apelin-13 (6 mmol/L, 0.1 μL) was microinjected into the rat PVN in the thoracic trauma group, and the effects of apelin-13 on the blood pressure (BP), heart rate (HR) and the discharge of rostral ventrolateral medulla (RVLM) neurons were observed through the simultaneous recording technology by polygraph. The results showed that the APJR mRNA expression was significantly decreased in the rats of trauma group as compared with that in the control group ($P < 0.05$), and a decline trend of apelin mRNA expression was also observed. EA application at bilateral Neiguan acupoints partially recovered the decline of APJR and apelin mRNA expression by the treatment of thoracic trauma. Both mean arterial pressure and HR in the thoracic surgical trauma group were significantly increased by the microinjection of exogenous apelin-13 into the PVN ($P < 0.05$), and the single-unit discharge rate of RVLM neurons also had an increasing trend. These results suggest that apelin in the PVN can improve the cardiac function of thoracic surgical trauma rats, and may be involved in the protective effects of EA.

Key words: apelin; surgical trauma; paraventricular nucleus; rostral ventrolateral medulla; electro-acupuncture

下丘脑室旁核apelin对手术创伤大鼠心功能的保护作用

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摘 要：Apelin是一种新型的内源性活性肽。本研究旨在探寻下丘脑室旁核(paraventricular nucleus, PVN) apelin是否能够改善开胸手术创伤大鼠的心功能，以及是否参与了电针的保护作用。将大鼠随机分为正常对照组、开胸手术创伤组和开胸手术创伤+电针内关组，采用荧光定量PCR法检测各组大鼠PVN区apelin及其受体(apelin receptor, APJR) mRNA的表达，然后通过多通道同步记录技术观察PVN微量注射外源性apelin-13 (6 mmol/L, 0.1 μL)对开胸手术创伤组大鼠的血压、心率以及延髓头端腹外侧区(rostral ventrolateral medulla, RVLM)神经元放电活动的影响。结果显示：与正常对照组比较，创伤组大鼠PVN区APJR mRNA的表达显著减少($P < 0.05$)，apelin mRNA的表达也有下降趋势；对开胸手术创伤大鼠施电针双侧内关穴后，PVN区APJR和apelin mRNA的表达水平又有所恢复。PVN微量注射外源性apelin-13可明显升高开胸手术创伤组大鼠的平均动

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脉压和心率($P < 0.05$)，RVLM神经元单位放电频率也有升高趋势。以上结果提示，PVN区apelin能够改善开胸手术创伤大鼠心功能，也可能参与了电针的保护作用。

关键词：apelin；手术创伤；室旁核；延髓头端腹外侧区；电针
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Apelin, a novel endogenous active peptide discovered recently, has a variety of structural forms, including apelin-13, apelin-36, *etc*. The precursor protein consisting of 77 amino acid residues is hydrolyzed to produce these active peptides of different lengths [1]. It is known that apelin and apelin receptor (APJR) are widely distributed in the cardiovascular system, gastrointestinal tract, other peripheral tissues and brain tissues (such as the hypothalamus, hippocampus, cerebral cortex, *etc*.) of human beings or rats [1–3]. Apelin and its receptor APJR participate in the regulation of various physiological functions, such as the cardiovascular function. Apelin can elicit a positive inotropic effect on the heart [4]. Pyroglutamyl apelin-13 ([Pyr^1] apelin-13), a more stable bioactive form of apelin-13 post-translational modification, can repair the function of ischemic myocardium by anti-remodeling and anti-myocardial apoptosis [5]. Apelin-13 can also salvage the peri-infarct region of myocardium to protect the cardiac function [6]. APJR may enhance Nodal/TGFβ signaling and contribute to cardiac development [7]. Apelin binding to its receptors may also play a role in lowering blood pressure (BP) by promoting nitric oxide (NO) production [8, 9]. These studies are related to the regulation of peripheral apelin and APJR on cardiovascular function, while the current mechanism of central apelin and APJR on cardiovascular regulation has not been fully elucidated. Reaux's team [10, 11] first reported the distribution of apelin-synthesizing neurons in the hypothalamus paraventricular nucleus (PVN) and supraoptic nucleus (SON) in rats, and the expression of APJR mRNA in SON vasopressin neurons. The abundant expression of APJR in the medial parvocellular and magnocellular regions of PVN suggests that apelin is involved in stress response [12]. Recent reports have revealed that apelin may have a protective effect on cardiovascular function [4–7].

Our previous studies showed that thoracic surgical trauma can reduce cardiac function, while electro-acupuncture (EA) application at bilateral Neiguan (PC 6) acupoints can improve cardiac function in surgical trauma-stressed rats[13, 14]. Can apelin in the PVN improve the cardiac function of thoracic surgical trauma rats? Does apelin participate in the protective effect of EA? These questions need further investigation.

Therefore, this experiment was designed to detect the mRNA expression of APJR and apelin in the PVN region by real-time PCR, and then observe whether exogenous apelin-13 applied into the PVN can improve the cardiac function of traumatic rats through the simultaneous recording technology by polygraph, and preliminarily explore whether apelin in the PVN participates in the protective effect of EA on cardiac function in surgical trauma rats.

1 MATERIALS AND METHODS

1.1 *Animal preparations*

Adult male Sprague-Dawley (SD) rats weighing 250–350 g were purchased from Qinglongshan Animal Breeding Grounds (Nanjing, China) and Shanghai Laboratory Animal Center (Shanghai, China). All experimental procedures conformed to guidelines of the Experimental Animal Ethics Committee of Wannan Medical College and Fudan University. Twelve rats were randomly divided into non-stressed group (control, $n = 4$), thoracic surgical trauma stressed group (trauma, $n = 4$), and bilateral Neiguan EA applied on thoracic surgical trauma stressed group (trauma + EA-PC 6, $n = 4$). Rats in each group were anesthetized with composite anesthetic agent (14 g urethane, 0.7 g chloralose and 0.7 g borax per 100 mL) by intraperitoneal injection, and then treated surgically. Trauma rat model preparation and EA process can refer to the previous study [13]. Trauma rat model was prepared by subjecting to a 4-cm-long left anterior thoracotomy and exposing thoracic cavity for 60 min, then the incision was sutured and the ventilator was removed. During the experiment, the arterial pH, pCO_2, and pO_2 were maintained within normal limits (pH: 7.35–7.45, pCO_2: 30–35 mmHg and pO_2: >100 mmHg). In the trauma + EA-PC 6 group, EA was applied on the bilateral Neiguan points for 30 min via the needles using a medical stimulator (0.5 ms, 5 Hz, G6805-2, China) during thoracotomy. The stimulation intensity (≤ 4 mA) was

just strong enough to cause slight twitches of the forelimb.

1.2 *Real-time PCR analysis for APJR and apelin mRNA expression in the PVN*

Rats were decapitated and the brains were removed with sterile instruments at 120 min after surgery. The brain tissue of the PVN (2 mm × 2 mm × 2 mm) was cut with a sterile blade on ice according to the atlas of the rat brain [15], and then the tissue was weighed and placed in a 5 mL centrifuge tube, frozen in liquid nitrogen and stored in a –70 °C refrigerator ready for real-time PCR. The total RNA was extracted with Trizol reagent. RNA concentration and purity were measured by a UV spectrophotometer (ND-1000, Nanodrop Technology, USA). Primers were synthesized as follows: the forward and reverse primers for GAPDH gene were 5'-GGAAAGCTGTGGCGTGAT-3' and 5'-AAGGTGGAAGAATGGGAGTT-3'; the forward and reverse primers for APJR gene were 5'-TGTACGCCAGTGTCTTTTGC-3' and 5'-GGATGTCAGTGGAACGGAAC-3'; the forward and reverse primers for apelin gene were 5'-CTGTTCTATTGCCGCTGGTT-3' and 5'-GCATCATAAAGTGGGAGTTGG-3'. RNA was reverse transcribed into first-strand cDNA. Real-time PCR was performed with the ABI 7900 system (Applied Biosystems, USA) at the following cycle: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 20 s, 72 °C for 20 s and 82 °C for 20 s, and fluorescence acquisition at 82 °C. GAPDH was a housekeeping gene used for normalization of the results. The relative concentration of the target genes was the ratio of the target gene concentration to the concentration of the housekeeping gene.

1.3 *The simultaneous recording by polygraph and PVN microinjection*

A polyethylene plastic catheter filled with 0.1% heparin sodium saline was inserted into the left femoral artery for measurement of BP, and then the catheter was connected to the transducer access to PowerLab multichannel bio-signal acquisition system (PowerLab 8/30, AD Instruments Inc., Sydney, Australia) via bridge amplifier (ML221, AD Instruments, Australia). Needle electrodes were inserted into the subcutaneous layer of the upper and lower limbs, and then the wire was connected to the other channel through the amplifier for recording the standard limb II lead electrocardiogram (ECG). As previously described [14], the rat head was fixed on the stereotactic apparatus (Model 68002, Shenzhen Ruiwo De Life Technology Co., Ltd., China), and the PVN (1.8 mm behind the Bregma, 0.4 mm left lateral to midline, and 7.9 mm below the skull surface) and the rostral ventrolateral medulla (RVLM, 12.00–12.36 mm behind the Bregma, 1.8–2.2 mm left lateral to midline, and 10.2–10.7 mm below the skull surface) were respectively located according to the atlas of Paxinos and Watson [15]. A burr hole was drilled over the PVN and RVLM, respectively, and the meninx was opened. A flat head microtiter containing saline or apelin-13 solution (6 mmol/L, 0.1 μL, Sigma-Aldrich) was placed vertically into the PVN via a brain stereotaxic apparatus. The dose of the microinjection of exogenous apelin-13 into PVN nuclei in the thoracic trauma group was determined according to the similar study [16]. The recording microelectrodes (filled with a 2% solution of Chicago sky blue in 0.5% sodium acetate, a resistance of 5–15 MΩ) were placed into the RVLM, and then, the electrodes were fine-tuned manually down to look for the spontaneous discharge of RVLM neurons. The recording signal was input to a channel of the PowerLab multi-channel bio-signal acquisition system via a microelectrode AC amplifier (Model 1800, A-M Systems, USA). Signals were recorded stably for 20 min before PVN microinjection. The data were analyzed later with Labchart software (AD Instruments Inc., Sydney, Australia). At the end of the experiment, microelectrophoresis of a 2% solution of Chicago sky blue was performed through recording electrodes [14], and then the brains were removed and fixed in 4% formaldehyde solution for 4–7 d. The coronal sections of brain were prepared, and the RVLM recording site and the microinjection site of PVN were observed and identified to determine whether the location was accurate according to the atlas [15].

1.4 *Statistical analysis*

The data were expressed as mean ± SEM. Comparison of mRNA expressions among different groups was performed by one-way analysis of variance (one-way ANOVA) with least significant difference (LSD) test. Paired *t* test was used to compare the parameters before and after administration. $P < 0.05$ was considered statistically significant.

2 RESULTS

2.1 Expression of APJR and apelin mRNA in the PVN

The expression of APJR and apelin mRNA in the PVN

region from four samples of each group were detected. The results showed that the APJR mRNA expression was significantly decreased in the rats of trauma group compared with that in the control group ($P < 0.05$), and a decline trend of apelin mRNA expression was also observed. In trauma + EA-PC 6 group, APJR and apelin mRNA expressions were partially recovered by the application of EA on Neiguan acupoints (Fig. 1).

2.2 Effects of apelin on the cardiovascular parameters of traumatic rats with thoracic surgery

The mean arterial pressure (MAP), heart rate (HR), and single-unit discharge rate of RVLM neurons were analyzed from the original traces before and after microinjection of apelin-13 solution or saline into the PVN. HR was analyzed from the ECG traces. The results showed that the MAP and HR were both significantly increased at 10 min after microinjection of apelin-13 into the PVN ($n = 9$, $P < 0.05$) (Fig. 2, Table 1). The single-unit discharge rate of RVLM neurons also had an increasing trend after administration. Twenty minutes after microinjection of apelin-13, the three parameters showed a restoring trend (Table 1). The results showed that microinjection of saline into the PVN had no significant effects on the MAP, HR and discharge rate of RVLM neurons. There was no significant difference before and after administration (Table 2).

At the end of the experiment, the 2% solution of Chicago sky blue was microelectrophoresed into the RVLM. The recording site and the location of the micro-syringe needle were observed to identify the

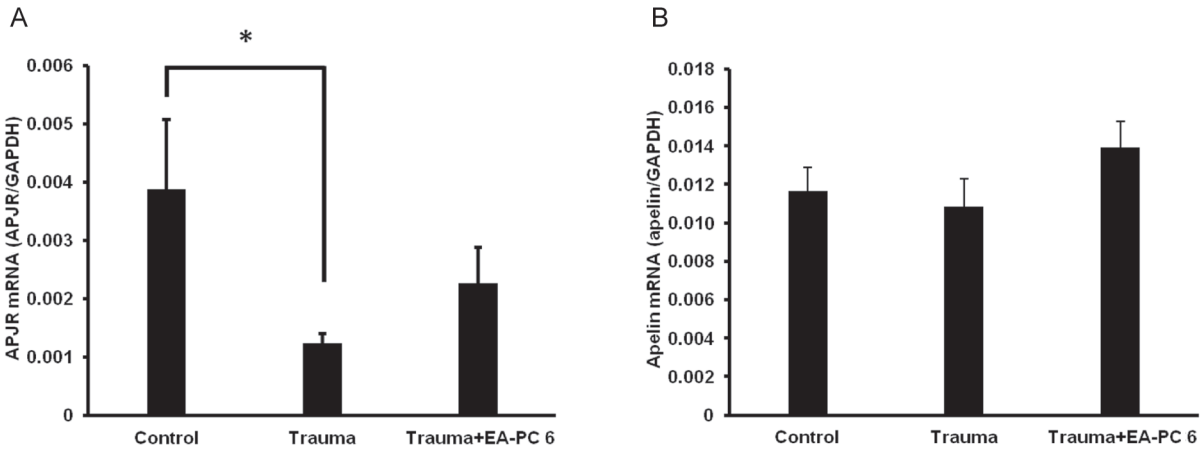

Fig. 1. The mRNA levels of apelin receptor (APJR, *A*) and apelin (*B*) in the PVN. Data represent mean ± SEM. One-way ANOVA with LSD test: $^{*}P < 0.05$. $n = 4$ in each group.

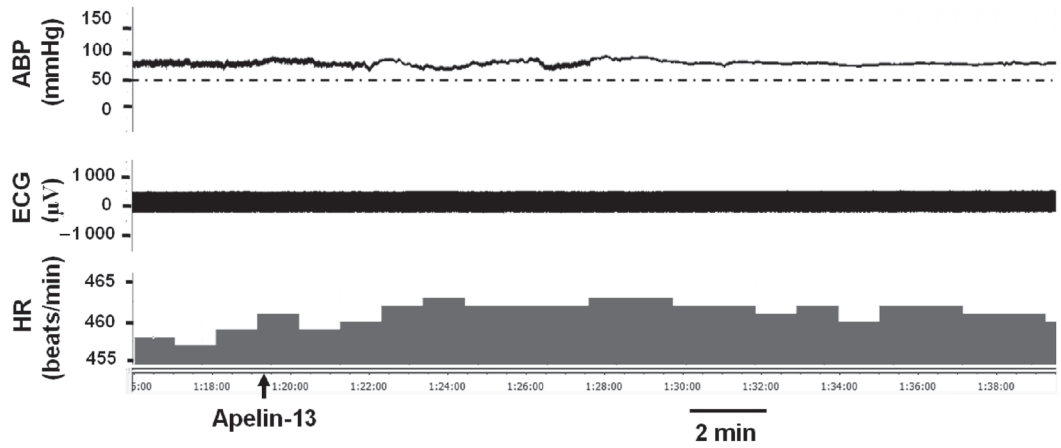

Fig. 2. The representative tracings of ABP, ECG and HR. The upward arrow indicates the start at which PVN microinjection of apelin-13 was given. The dotted line shows the 50 mmHg level. The trace of HR is the histogram of ECG. ABP: arterial blood pressure; ECG: electrocardiogram; HR: heart rate.

Table 1. The changes of the cardiovascular parameters before and after apelin-13 microinjection into the PVN

	Pre-microinjection	10 min after microinjection	20 min after microinjection
MAP[a] (mmHg)	85.9 ± 2.7	89.3 ± 2.1*	86.3 ± 2.1
HR[a] (beats/min)	372.5 ± 13.8	377.7 ± 13.3*	373.4 ± 13.6
Discharge rate[b] (Hz)	8.5 ± 4.0	9.1 ± 4.0	8.3 ± 4.4

Data represent mean ± SEM. [a]: $n = 9$; [b]: $n = 5$. Paired t test: *$P < 0.05$ *vs* Pre-microinjection. Discharge rate is the frequency of the single-unit discharge of RVLM neurons. MAP, mean arterial pressure; HR, heart rate.

Table 2. The changes of the cardiovascular parameters before and after saline microinjection into the PVN

	Pre-microinjection	10 min after microinjection	20 min after microinjection
MAP[a] (mmHg)	82.0 ± 3.8	84.8 ± 6.4	85.4 ± 6.4
HR[b] (beats/min)	375.2 ± 13.8	371.6 ± 15.3	368.1 ± 15.1
Discharge rate[a] (Hz)	8.6 ± 2.0	8.9 ± 2.1	8.2 ± 1.9

Data represent mean ± SEM. [a]: $n = 4$; [b]: $n = 6$. Discharge rate is the frequency of the single-unit discharge of RVLM neurons. MAP, mean arterial pressure; HR, heart rate.

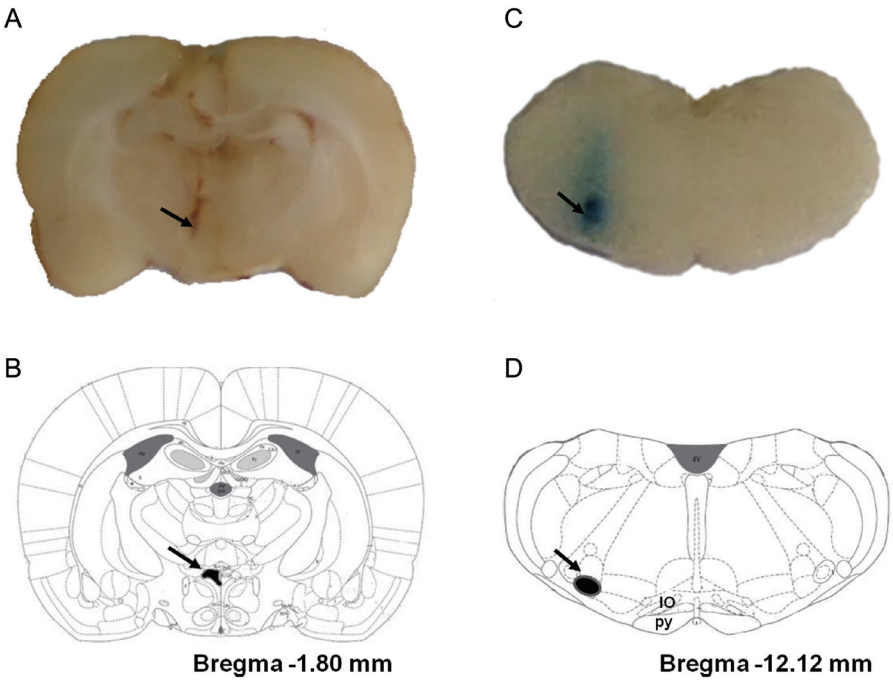

Fig. 3. Localization of the recorded and microinjected nuclei. *A*: A photograph of hypothalamic coronal slice. *B*: The diagram containing PVN from atlas (1.80 mm behind the Bregma). *C*: A photograph of brain stem coronal slice. *D*: The diagram containing RVLM from atlas (12.12 mm behind the Bregma). The arrows indicate the PVN (*A*, *B*) or the RVLM (*C*, *D*), respectively. py, pyramidal tract; IO, inferior olivary nucleus.

accuracy of the recorded and microinjected nuclei localization, and the photographs of the typical coronal slices were shown in Fig. 3.

3 DISCUSSION

In this study, the mRNA expression of APJR and apelin were detected by real-time PCR in the PVN of three groups (control, trauma and trauma+EA-PC 6 groups), and the results confirmed that this novel endogenous active peptide apelin and its receptor APJR are indeed distributed in the rat hypothalamus [10, 11]. In particular, it is more interesting to note that the APJR mRNA expression of the traumatic group was significantly decreased, and the expression of apelin mRNA of the traumatic group also had a decline trend, as compared with those in the control group.

In our previous study [13], it was observed that the cardiac

function was declined in traumatic rats. From the results in the present study, we speculate that apelin and APJR may be involved in this regulation of cardiac function. More specifically, the decrease in cardiac function of traumatic rats may be related to the reduction of apelin-APJR system function. It has been reported that peripheral apelin can lower BP, but the central apelin can raise BP [17], for example, intracerebroventricular injection of [Pyr^1] apelin-13 dose-dependently increased MAP and HR [18]. Therefore, in this experiment, the reduction of apelin-APJR system function in the PVN area of traumatic rats may lead to decreases of BP and HR. So, will the upregulation of the apelin and APJR expression enhance the cardiac function of traumatic rats? In order to answer the question, microinjection of exogenous apelin-13 into the PVN was also carried out to observe the effect of apelin. As a result, it was observed that exogenous apelin-13 could significantly increase the MAP and HR in thoracic trauma rats, which further suggests that the expression upregulation of apelin in the PVN may indeed play a role in improving cardiac function in traumatic rats. What can be done to recover the expression of apelin and APJR in traumatic rats?

Our previous work showed that Neiguan EA could significantly improve the cardiac function of traumatic rats [13]. Neiguan is an important acupoint to protect cardiac function. It has also been reported that repetitive EA stimulation at the Zusanli (ST 36) acupoint attenuated hypertension in stress-induced hypertension rats [19]. Quchi (LI 11) EA stimulation can also down-regulate arterial blood pressure (ABP) in hypertension rats [20]. It indicates that other acupoints also have a protective effect on cardiac function, such as Zusanli, Quchi acupoints, and so on. However, there are many acupoints that do not participate in regulating cardiovascular activity, such as "Lieque (LU 7)" acupoint. Our previous experiments also support this view [13]. This allows us to consider whether EA can improve the cardiac function by restoring the expression of apelin and APJR in the traumatic rats. It was indeed observed that Neiguan EA partially recovered the expression levels of both genes in the trauma+ EA-PC6 group in the present study. However, this also needs to be further verified.

At the same time, the role of apelin-13 in the PVN increasing BP was further explored by simultaneous observation of RVLM neuronal discharge activity. The pre-autonomic neurons of the PVN project to RVLM and spinal cord, and regulate the sympathetic output [21]. These neurons may be the neurons that synthesize arginine vasopressin (AVP), corticotropin releasing hormone (CRH), angiotensin II or glutamate. Thus, apelin-13 in the PVN region may activate the RVLM cardiovascular center by acting on these pre-autonomic neurons, and then increase BP and HR by enhancing sympathetic nervous system activity. The cardiac sympathetic tone and sympathetic vasoconstrictor tone originated from the RVLM. In this experiment, after apelin-13 microinjection into the PVN, the MAP and HR rise were indeed observed, and the discharge activity of RVLM neurons recorded simultaneously also had an increasing trend. Zhang *et al*. [16] reported that apelin and APJR in the PVN region of spontaneously hypertensive rats may also increase BP by activating sympathetic activity and promoting AVP release. Therefore, we speculated that there may be other factors involved in elevating the BP and HR of traumatic rats when exogenous apelin-13 was microinjected into the PVN. For example, apelin-13 may stimulate AVP release from magnocellular neurons of PVN [11, 22] or promote CRH release by activating the hypothalamus-pituitary-adrenal (HPA) axis [23], which can improve the amount of norepinephrine, adrenaline or other hormones in peripheral blood. That is, the apelin-13 in the PVN regulates cardiovascular function by means of neurohumoral regulation. Of course, these also need to be further confirmed.

Although APJR was also observed to be reduced in traumatic rats, exogenous apelin-13 may also raise BP and HR by acting on other receptors. It has been reported [24] that [Pyr^1] apelin-13 mediates the effects of hypertension by acting on the vasopressin V_{1a} receptor when [Pyr^1] apelin-13 was microinjected into the RVLM. Thus, apelin in the PVN region may also play a role by acting on other receptors, which needs to be further demonstrated.

In summary, surgical trauma can weaken the function of apelin-APJR system, which may also be involved in the decline of cardiac function in traumatic rats. The increases of MAP and HR can be induced by exogenous apelin-13 microinjection into the PVN. Neiguan EA can reverse the reduction of APJR and apelin mRNA expression in PVN region of thoracic surgical trauma rats to a certain extent. The results suggest that apelin in the PVN can improve the cardiac function of thoracic surgical trauma rats, and may be involved in the protective effects of EA.

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