Original Article
Expression of oxytocin receptor in the rat superior cervical ganglion after myocardial infarction

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Abstract: Background: Myocardial infarction (MI) accompanied with abnormal sympathetic innervation, meanwhile, some studies have revealed the oxytocin (OT) and its receptor (OTR) have a relationship with MI and sympathetic system. It is assumed that OT has an close relationship with superior cervical ganglion (SCG), but the existence of oxytocin receptors in SCG has not been well clarified. Objective: Our research aims to explore the expression of OTR in SCG in the setting of MI. Methods and results: MI was induced by coronary artery ligation. Rats were randomly assigned to 2 groups: control, MI. The expression of OTR was measured by Western blotting. Distribution of OTR in SCG was investigated by immunofluorescence. Retrograde tracing test revealed the sprouting of tyrosine hydroxylase (TH: the markers of sensory afferent fibers) from cardiac to SCG neurons. The double-immunofluorescence evidence showed that OTR was co-localized and concomitantly changed with TH and the retrograde neuronal labeling from the cardiac afferent nerves. By Western blotting, the protein of OTR in the MI group was higher than those of the control group. Conclusions: The expression of OTR in SCG after experimental myocardial infarction group was enhanced, suggesting the involvement of OTR in SCG may play a role in the transmission of sympathetic responses after MI.

Keywords: Oxytocin receptor, superior cervical ganglion, myocardial infarction

Introduction

The OTR belongs to the class A (rhodopsin-like) super-family of G protein coupled receptors (GPCRs). The oxytocin (OT) -oxytocin receptor (OTR) system has been known for its pivotal role in many aspects of mammalian reproduction as well as several other physiological processes such as bond pairing and cardiovascular homeostasis. It has been reported that both OT and OTR are found in the heart [1] and OT plays an important role in the regulation of cardiovascular function [2]. In addition, some investigators have provided evidence that oxytocin may be involved in regulation of the cardiovascular system by direct peripheral and indirect central actions [3-6]. Furthermore, OT has been reported to facilitate wound healing [7]. The above data suggests that OT involves in the pathogenesis of MI.

Previous work showed that the up-regulation of transmitter receptors in the stellate ganglia (SG) and SCG after MI injury resulted in increased blood pressure and HR by strengthening the activity of the sympathetic post-ganglionic neurons [8]. Clinical research found that removal of the cervical sympathetic ganglia, such as SCG or SG, causes the symptoms of angina pectoris disappear in 50-60% patients with coronary heart disease [9]. Electric stimulation of the SCG causes an increase in OT release and 20 days after SC ganglionectomy the OT release increased [10]. It is assumed that OT has a close relationship with SCG, but the existence of oxytocin receptors in SCG has not been well clarified.

Many studies have revealed ventricular arrhythmia is one of the most important causes of death in patients with MI and sympathetic activation plays an important role in the pathogenesis and maintenance of arrhythmia in both patients and animal models of MI [11-14].

Oxytocin terminal fibers are closely associated with sympathetic preganglionic neurons (SPNs). OT fibers make synaptic contact with SPNs pro-

jecting into the SCG [15]. The present study was undertaken to verify the functional oxytocinergic receptors were co-localized with the SPNs.

Materials and methods

Animals and experimental protocol

Adult male Sprague-Dawley rats (200-250 g, Vital River, Beijing, China) were used. They were housed in separate cages (2-5 rats per cage) floored with wood shavings and maintained in the temperature-controlled room (22±2°C) with a dark-light cycle of 12 h/12 h. All procedures were carried out according to approved protocols and guidelines established by the Shandong University Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals and their suffering. 24 rats were enrolled and randomly divided into two groups: the normal control group (NC group, n=8), and experimental myocardial infarction group (MI group, n=16). The rats were sacrificed then the tissues were snap-frozen and stored separately at -80°C for further biochemical analysis or were fixed in 10% zinc formalin or embedded in OCT for histological examination.

Experimental myocardial infarction

After a 7-day acclimatization period, open-chest MI surgery was performed. Each rat was anesthetized with an intraperitoneal injection of 30 mg/kg of 3% sodium pentobarbital (Sigma-Aldrich, St. Louis, Mo., USA), the depth of anesthesia was monitored by observing blood pressure, heart rate and the absence of corneal and paw pinch reflexes. Then rats were intubated via tracheotomy, and connected to a small-animal respirator (HX-300S, TME, Chengdu, China), with an adjusted rate of 30-40 breaths/min and a tidal volume set to 1.1-1.3 ml/100 g body weight. A small area of skin overlying the left thoracic wall was shaved and prepared for surgery. An incision was made in the skin, parallel to the rib in the region overlying the fifth or sixth intercostal space. Muscle layers were divided, a small incision was made in the pleura then the heart was exposed, and the left coronary artery was ligated 2-3 mm from its origin between the pulmonary artery cones and the left atrium with a 6-0 polypropylene ligature as previously described [16]. A

constant body temperature of 37°C was maintained with a heating pad. Muscle and skin was sutured and the animal was removed from the respirator and allowed to recover in a warm environment. The animals were closely monitored during the recovery period. The infarction was confirmed by ST segment elevation, regional cyanosis, and wall motion abnormalities (see Figure S1). With respect to clinical importance, only rats with moderate infarct size (30% to 50%) were enrolled. The rats received postoperative daily intramuscular administration of penicillin (800,000 U, NCPC, China) for 3 days.

Cardiac injections of WGA-HRP

An intrathoracic approach was used to inject the tracer. The anatomical process was similar with above. WGA-HRP (Sigma, St. Louis, Mo., USA) was dissolved in 0.9% normal saline and injected with a 30-gauge needle connected to a Hamilton syringe through high-pressure tubing. WAG-HRP (20 μL, diluted in 0.9% normal saline) was injected into cardiac apex and cones arteriosus at five points by a microinjector. Following a period of 7 days to allow the retrograde, transport of the WGA-HRP, the animals were euthanize and the samples were collected.

Immunofluorescence

SCGs were immersed in 30% sucrose in PBS overnight, embedded in Tissue-Tek® OCT compound (Sakura Finetek), and frozen in an isopentane bath on dry ice. Samples were incubated with anti-TH Ab (1:2400; Millipore), anti-OTR Ab (1:500; Abcam) overnight at 4°C followed by a two-hour incubation with FITC-conjugated rabbit anti-sheep (1:200; Bethyl), Alexa 545-conjugated goat anti-rabbit (1:100; Peprotech) or FITC-conjugated rabbit anti-mouse (1:200; BioLegend) secondary antibodies. The sections were counterstained with DAPI (Life Technologies) to identify nuclei. For quantification of the fraction of sympathetic nerve fibers, we analyzed four sections adjacent to those stained with Masson's trichrome; the first section used for analyses was taken 1.5-2 mm apical to the ligation [17].

qRT-PCR

Total RNA was isolated from SCG with TRIzol reagent (Invitrogen, CA, USA). Candidate gene
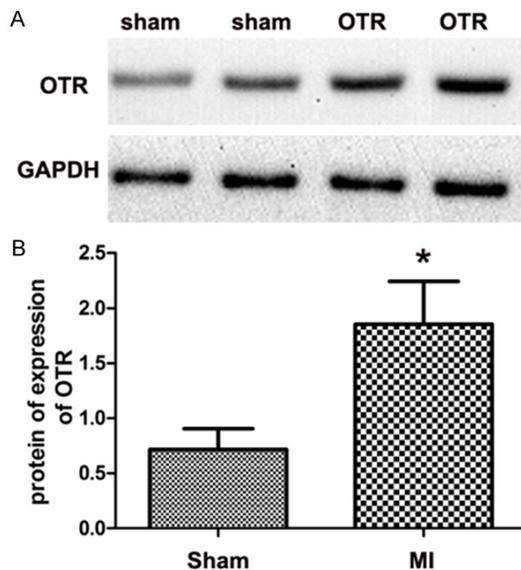
Figure 1. Expression profile of OTR after myocardial infarction (MI). A. Western blot showing the expression profiles of OTR in the left ventricle post-MI. B. OTR was quantified relative to the GAPDH (37 KD) levels. *P<0.05 vs. sham group.

expression was measured by quantitative real-time PCR using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). Each measurement was performed with gene-specific primers and SYBR Green 1 using a Bio-Rad iQ5 Multicolor Real Time PCR machine (Bio-Rad Laboratories). For each sample, both GAPDH and the target gene were amplified in triplicate in separate tubes. A relative quantification $2^{-\Delta\Delta CT}$ method was used for comparison between groups. The primers for each gene used in this study were as follows: OTR: F: 5'-CGATTG CTG GGC GGT CTT-3', R: 5'-CCG CCG CTG CCG TCT TGA-3'; GAPDH: F: 5'-AGATCCACAACGGATACATT-3', R: 5'-TCCCTCAAGATTGTCAGCAA-3'.

Western blot analysis

For immunoblot analyses, proteins were isolated using a Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Extracted protein was measured with the BCA protein assay reagent kit (Pierce). Equal amounts of total protein (80 µg of protein/lane) were resolved on 5-10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk in PBST (PBS containing 0.05% Tween 20), and incubated overnight at 4°C with primary antibodies

against OTR (Abcam, 1:600). The blots were developed using an enhanced chemiluminescence (ECL) detection kit (Millipore) and visualized using a FluorChem E Image (Protein-Simple, Santa Clara, CA, USA). The densities relative to GAPDH were analyzed using NIH Image software.

Statistics

Data are presented as the means ± standard deviations (SD). Unpaired t-tests were used to compare values between two groups. Analyses were performed using SPSS 17.0 software (SPSS Inc. Chicago, IL, USA). *P*-values <0.05 were considered statistically significant.

Results

OTR expression in the SCG

Firstly, we aimed to explore the expression of OTR in SCG from MI rats and normal rats using Western blotting analysis. The protein level of OTR is shown in **Figure 1**. As a result, the OTR expression is significantly increased at 7 days post infarction when compared with normal rats (*P*<0.05).

Immunofluorescence labeling of OTR and TH immunoreactivities

Following injections of WGA-HRP into the left ventricle wall, retrogradely labeled tracer were observed in both left and right SCG. The immunoreactivities of OTR and TH were detected in the SCG with double-staining immunofluorescence in rats of 7 days of MI to exam the colocation of OTR and TH in the ganglia. OTR positive cells presented red fluorescence (**Figure 2**), TH was stained as green fluorescence (**Figure 2**). We observed that the presence of OTR throughout the confines of the SCG were extensive and double immunolabeling with OTR and TH showed the OTR was expressed in SCG. The double-label immunofluorescence of OTR and TH in MI group exhibited higher density than that in sham group. Co-expression value (integrated optical density, IOD) of OTR and TH in the SCG (*P*=0.006, *n*=6) of MI group increased significantly in comparison with that in control group (*P*<0.05, *n*=6).

WAG-HRP was used as a retrograde tracing marker to observe the retrograde neuronal
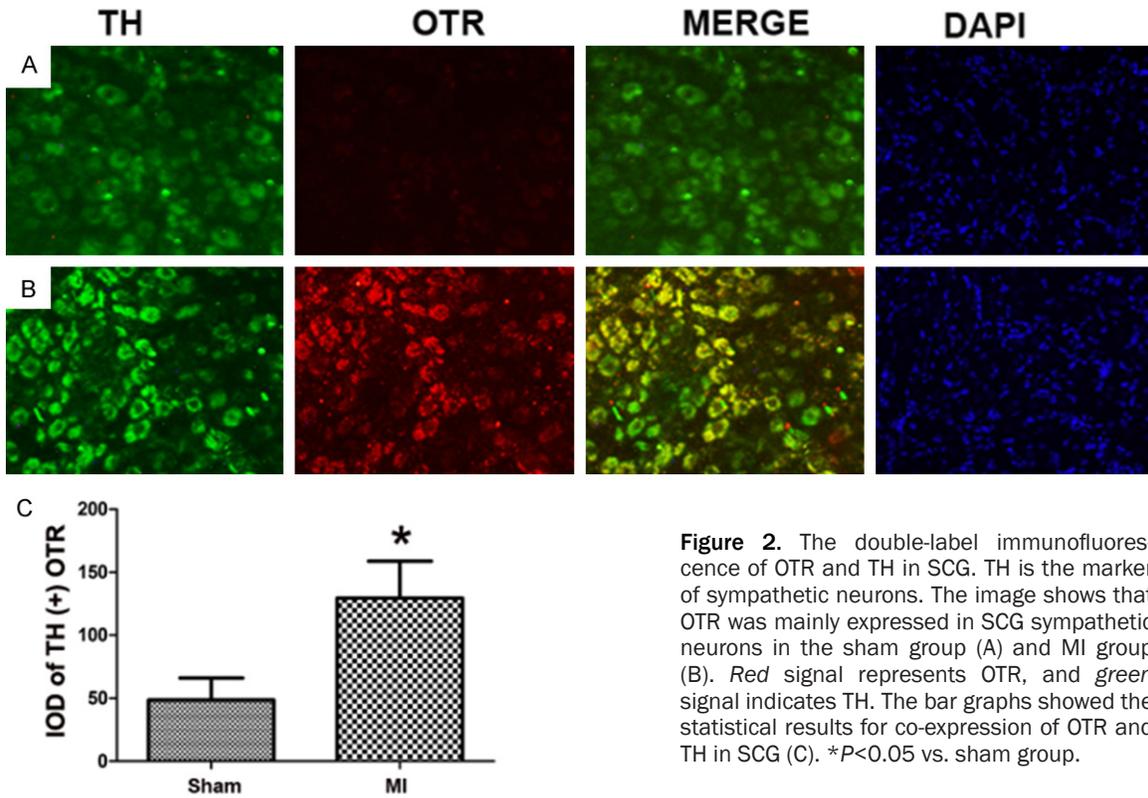
Figure 2. The double-label immunofluorescence of OTR and TH in SCG. TH is the marker of sympathetic neurons. The image shows that OTR was mainly expressed in SCG sympathetic neurons in the sham group (A) and MI group (B). *Red* signal represents OTR, and *green* signal indicates TH. The bar graphs showed the statistical results for co-expression of OTR and TH in SCG (C). *P<0.05 vs. sham group.
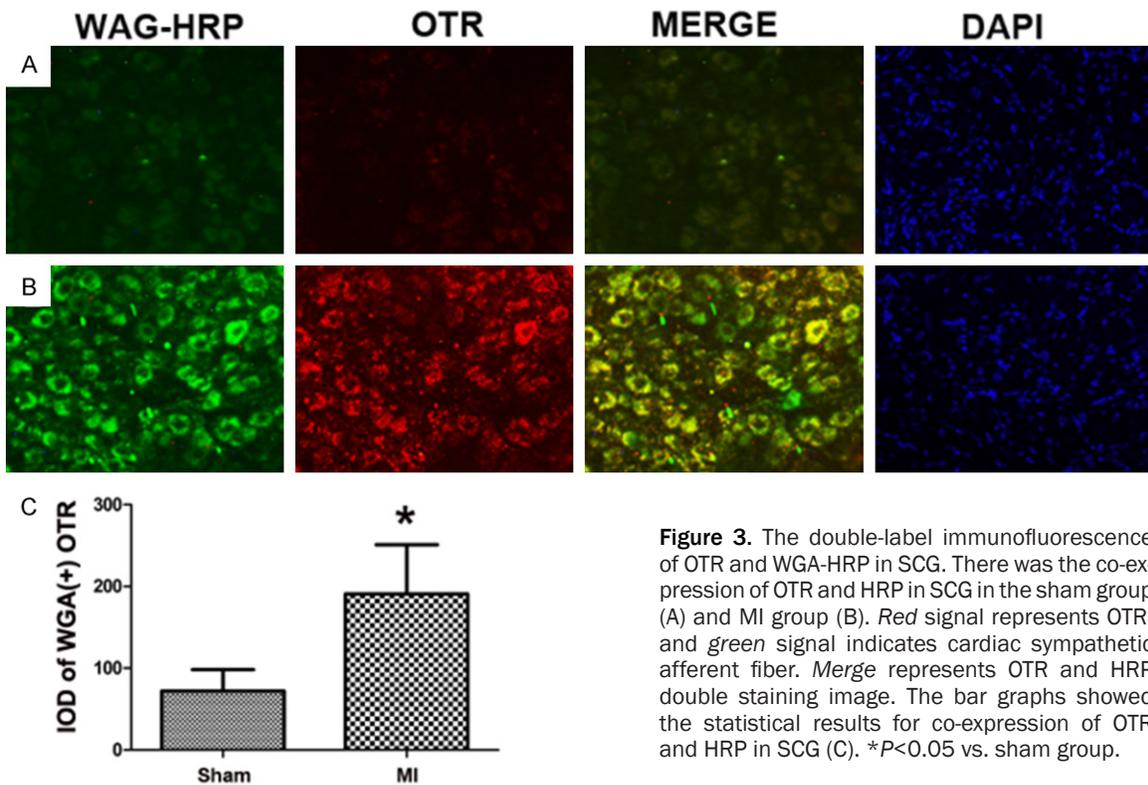
Figure 3. The double-label immunofluorescence of OTR and WGA-HRP in SCG. There was the co-expression of OTR and HRP in SCG in the sham group (A) and MI group (B). *Red* signal represents OTR, and *green* signal indicates cardiac sympathetic afferent fiber. *Merge* represents OTR and HRP double staining image. The bar graphs showed the statistical results for co-expression of OTR and HRP in SCG (C). *P<0.05 vs. sham group.

labeling from the cardiac afferent endings to SCG. Sham control and MI rats, were also studied. In MI group, WAG-HRP was injected into cardiac apex and conus arteriosus at 7 days after MI operation. The double immuno-fluorescence labeling of HRP and OTR in the SCG of sham group was observed 7 days later, which displayed barely staining. By contrast, the staining of OTR and HRP by double immunofluorescence labeling in MI group was more intense (*n*=6, respectively; *P*<0.05), suggesting the neuronal labeling of OTR receptor from the cardiac afferent ending to SCG (**Figure 3**).

Discussion

In our present study, we can see that after the MI, OTR in SCG activated and its expression level of protein up-regulated in SCG and made the SCG postganglionic neurons excited. We presume that OTR was involved in the transmission of sympathetic excitation after the myocardial ischemic infarction and decrease sympathetic activity via inhibiting OTR in rat SCG to protect the myocardium. These findings would offer potential target for AMI therapy.

OT, as a neurohypophysial neuropeptide involved in the regulation of lactation and parturition, it and its receptor (OTR) are traditionally known for their important role in female reproductive functioning, including uterine contraction and milk ejection, but have also been shown to be expressed locally within vascular and cardiac tissues in rats and humans [18-21]. The regulatory spectrum of OT is now known to be substantially wider. More and more data strongly indicates that oxytocin may participate in the control of affecting behavior, cognitive functions, and stress-related reactions [22-29].

OT has also been identified as a cardiovascular hormone [30]. The transcription and expression of the OTR is tightly regulated within cardiovascular tissues. OT synthesis and receptors are reported in both cardiac and vascular tissue in non-human mammalian species [19, 20], recent observations obtained by one research group indicate that OT may induce cardioprotective effects [31]. OT acts both centrally and peripherally at multiple sites including brain stem, heart, and vessels to exert acute and long-term inhibitory effects on cardiovascular activity [21, 32-39]. Similarly, OT administered in vitro reduces the rate and force of cardiac cells' intrinsic contractions causing them to 'beat' more slowly and contract less forcefully [40]. Findings from both animal and human studies suggest that plasma OT may reduce physiologic stress responses. It has been proposed that OT may play a role in mediating the beneficial effects of affiliate social interactions on atherosclerosis [41]. It is also shown that the role of endogenous OT in buffering the cardiovascular responses to stress is impaired in rats after MI [42]. Some results indicated that the OTR could significantly protect gastric mucosal against injury induced by ischemia-reperfusion, and the OTR was involved [43]. So we suppose that the OTR may exert a cardiovascular protective effect. Postinfarct treatment with OT has been shown to reduce myocardial infarct size and improves LV function and remodeling by activating OT receptors and pro-survival signals and by exerting antifibrotic and angiogenic effects through activation of MMP-1, endothelial NO synthase, and vascular endothelial growth factor [44]. These findings provide new insight into therapeutic strategies for ischemic heart disease.

The sympathetic innervation of the rat heart after MI was investigated by retrograde neuronal tracing and multiple label immunohistochemistry. Injections of WGA-HRP made into the left ventricular wall labeled sympathetic neurons that were located in SCG, then confirm the existence of this link between heart and SCG. Cervical sympathetic ganglia were not only a simple relaying station for signals, but also an integration center for all kinds of neurotransmission [45-47].

The relationship between OT and sympathetic activity has more and more been focused. OT has been implicated in autonomic regulation with a direct action on SPNs [48-51]. Indirect evidence in humans is the observation that OT might be a partial mediator of the attenuating effect of partner support on resting levels of circulating noradrenaline in women [52]. Thus, further investigation of the involvement of OT in sympathetic and adrenomedullary systems is warranted. Electron microscopical observations showed that oxytocin-immunoreactive terminals form synapses with dendrites or

soma of the SPNs. These results clearly show that a large proportion of SPNs are innervated by oxytocin-containing fibers.

A population of PVN-spinal OT neurons excite cardiac sympathetic preganglionic chronotropic neurons controlling heart rate [53]. High concentrations of OT binding sites have been found in the autonomic areas of the thoracic and upper lumbar spinal cord and in close association with SPNs [48]. Oxytocin-like immunoreactive axons have been shown to form synaptic contacts with dendritic profiles in the autonomic areas [54]. Since activation of autonomic SPNs in the thoracic spinal cord produces pupillary dilatation, we propose that OT is a central nervous system neurotransmitter that stimulates these neurons directly, or perhaps indirectly, and thus is a mediator of VS-produced pupillary dilatation [55]. It is possible that OT receptors in sympathetic afferents were triggered during MI.

The SCG not only transmit the sympathetic preganglionic signal but also have the integration effect in the regulation on autonomic function [56]. The cervical sympathetic ganglia receive the afferent signals of visceral sensory endings and control the efferent signals of cardiac activity, forming the local feedback loops [47, 57, 58]. There were feedback loops in SCG between the cardiac sensory afferent nerves and the cardiac sympathetic postganglionic efferent neurons to modulate the postganglionic efferent effects [46, 47, 59-64]. It showed there is a clear coincidence of the location of preganglionic neurons in the spinal cord identified by retrograde tracing from the SCG in the rat [65]. In the present study, the simultaneous application of a retrograde tracer and OT immunocytochemistry showed at the light-microscopic level that SPNs projecting to the SCG were in apposition with OT-containing fibers. Therefore, OT receptors in the rat cervical sympathetic ganglia may be involved in the transmission of cardiac nociception.

Sympathetic postganglionic fibers form cardiac plexus innervating myocardial tissues. Nociceptive afferent signals of myocardial ischemic injury can lead to the excitement of cardiac sympathetic efferent nerves through the SCG. The sympathoexcitatory reflex leaded to an increase in blood pressure and heart rate, which aggravated the myocardial ischemic injury [60, 66-68]. Several types of transmitter receptors in cervical sympathetic ganglia were involved in the transmission of myocardial ischemic nociceptive signals [60, 69, 70].

Our study indicates that OTR in SCG activated and expressed more after MI, after that how to explain this phenomenon? What's the exact role of OTR in the transmission of sympathoexcitatory after MI? Above questions need further research. Through review previous studies, We find two viewpoints: on one hand, several proinflammatory cytokines are known to play a role in the pathogen of atherosclerosis, the most notable of these being interleukin (IL)-1, tumor necrosis factor, and IL-6 [71]. OTRs are present on adipocytes and circulating monocytes and macrophages, but no studies have examined whether OT has an anti-inflammatory effect on visceral adipose tissue depots in vivo [18, 41]. Research from our laboratory showed that OT reduces NAD(P)H-oxidase activity and inflammatory cytokine secretion in stimulated macrophages and endothelial cells in vitro. This suggests that direct anti-inflammatory and antioxidant actions of OT on the endothelial surface and on macrophages could be responsible for the observed decrease in atherosclerosis in OT-treated animals. Future studies examining markers of vascular oxidative stress (e.g., NAD (P) H-oxidase activity) and inflammation after chronic OT administration may further clarify the mechanism by which OT affects lesion formation. On the other hand, Activation of the intrinsic OT system within the heart causes negative inotropic and chronotropic effects through an atrial natriuretic peptide-dependent pathway [18-20]. We are going to perform further study on the possible roles of OTR in the SCG mediated sympathoexcitatory responses in AMI rat using targeted OTR silencing.

Disclosure of conflict of interest

None.

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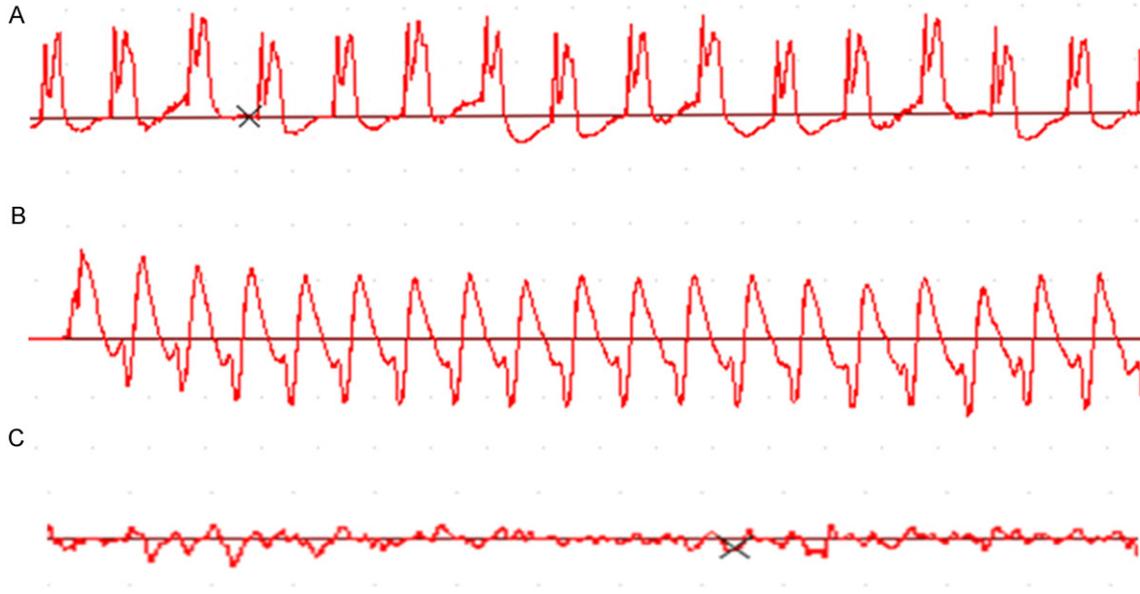
Figure S1. The manifestation of ECG after myocardial infarction. A. ST segment elevation. B. Ventricular tachycardia. C. Ventricular fibrillation.