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Involvement of the orexin system in sympathetic nerve regulation

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ABSTRACT

Orexin, also known as hypocretin, is a secreted neuropeptide implicated in the regulation of sleep and food intake. In the present study, we examined the importance of orexin in regulation of the sympathetic nervous system using an orexin/ataxin-3 transgenic (OXTg) rat, which has a minimal number of orexin neurons.

RT-PCR analysis identified expression of prepro-orexin and orexin receptor-1 (OX1R) in the superior cervical ganglion (SCG), and expression of another receptor (OX2R) was marginal in the wild-type rat. The orexin/ataxin-3 transgenic rat showed increased expression of OX1R and OX2R, whereas expression of prepro-orexin was undetectable, suggesting a compensatory increase in both receptors. In the ECG recording (R–R interval), orexin/ataxin-3 transgenic rats showed decreased responsiveness to the β-adrenergic blocker propranolol. Furthermore, OXTg rats had deteriorated R–R interval regulation, indicating involvement of the orexin system in sympathetic nerve regulation. This was accompanied by decreased baroreflex and responsiveness to β-adrenergic blocker in blood pressure recording, also suggesting involvement of the orexin system in sympathetic nerve regulation. Histological examination revealed hypotrophic changes in the transgenic heart, suggesting involvement of the orexin system in cardiac development. Taken together, our present results indicate involvement of the orexin system in sympathetic nerve control.

1. Introduction

Orexin-A (hypocretin 1; 33 amino acids; MW = 3562 Da) and orexin-B (hypocretin 2; 28 amino acids; MW = 2937 Da) have received attention as central nervous system (CNS) regulatory peptides involved in food intake and sleep behavior [1,2]. Both orexin-A and -B nerve fibers project widely into the brain, particularly throughout the hypothalamus and are implicated in the control of sleep and wakefulness [3] and cardiovascular function [4]. The loss or dysfunction of hypocretin neurons results in the sleep disorder narcolepsy [3], which is characterized by excessive daytime sleepiness, and sleep fragmentation.

The central effects of orexin peptides are mediated by G-protein-coupled receptors known as orexin receptor-1 (OX1R; 425 amino acids) and orexin receptor-2 (OX2R; 444 amino acids). Expression of orexin receptor mRNA is distributed extensively in the rat brain [5]. Within the hypothalamus, OX1R mRNA is most abundant in the ventromedial hypothalamic nucleus [5]. In contrast, OX2R mRNA exists mainly in the paraventricular nucleus (PVN) [5], which is involved in the integration of the autonomic nervous and neuro-endocrine systems.

Orexin neurons in the hypothalamus project to cardiovascular regulatory centers in the hindbrain including the nucleus tractus solitaries (NTS) and nucleus ambiguus, suggesting the involvement

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of the orexin system in blood pressure and heart rate regulation [6]. The role of orexin in regulation of blood pressure is supported by data from orexin knockout mice, which have low basal blood pressure [7].

Although the functions of orexin and orexin receptors have been characterized primarily in the CNS, substantial data indicate that orexins may function outside the CNS [8]. In our previous study, Nemoto et al., clarified expression of orexin and its receptors in bovine adrenal gland, which is a component of the sympathetic nervous system [9]. Although the relationship between orexin and the autonomic cardiovascular system has been suggested, the details of this relationship, such as effect of orexin on heart rate variability (HRV), have not been well characterized.

In the present study, we analyzed the involvement of the orexin system in cardiovascular autonomic regulation using the orexin/ataxin-3 transgenic rat (OXTg), which has a minimal number of orexin neurons.

2. Material and methods

2.1. Orexin/ataxin-3 transgenic rat

The *orexin/ataxin-3* transgene expresses an N-terminally truncated human ataxin-3 protein containing a Q77polyglutamine stretch under control of the human *prepro-orexin* promoter [10]. All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Hirosaki University School of Medicine.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Poly(A)$^+$ RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and Oligotex-dT30 (Takara, Shiga, Japan). The reverse transcription reaction was performed using a first-strand cDNA synthesis kit (SuperScript II Reverse Transcriptase, Invitrogen). PCR amplification was performed using GoTaq Green Master Mix (Promega, Madison, WI USA). Specific sequences of prepro-orexin, OX1R, OX2R, β-adrenergic receptors (β1 and β2), and β-actin were amplified by PCR. More detailed information is included in the supplemental information.

2.3. Western blot analysis

Partially purified cell membranes were prepared. Aliquots of homogenate (~7.0 μg) were resolved by 7.5% or 12% SDS-polyacrylamide gel electrophoresis. Commercially available polyclonal antibodies specific for orexin receptor-1 and -2, prepro-orexin were used. As a control, an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used. Goat polyclonal antibodies against prepro-orexin, OX1R, and OX2R, and a mouse polyclonal antibody against GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. General anesthesia

When the rats were 12–16 weeks old, anesthesia was induced by placing the rats in an anesthesia induction chamber (25 × 25 × 14 cm) containing 4% isoflurane (Forane; Abbott Japan Co., Ltd.; Tokyo, Japan) and room air. Subsequently, anesthesia was maintained using 2% isoflurane inhalation anesthesia (2 L/min) for ECG recordings. All experiments were conducted between 10:00 am and 4:00 pm.

2.5. Evaluating electrocardiogram (ECG)

ECG recordings, heart rate (HR), and R–R interval were measured simultaneously (ML846 Power Lab system, AD Instruments; Dunedin, New Zealand) [11]. An M-button (MB) connector was used for the connecting electrode [11]. Heart-rate variability (HRV) is an indicator of cardiac autonomic nerve control. For pharmacological analyses, rats were administered either propranolol (β-adrenergic blocker, 1.0 mg/kg) as a sympathetic blockade or atropine (0.5 mg/kg) as a parasympathetic blockade. To observe the baroreflex responses, carotid arteries were ligated for 30 s with 6–0 silk sutures. Detailed information is included in the supplemental information.

2.6. Blood pressure measurement

The arterial blood pressure was obtained using arterial catheters surgically inserted into the right carotid artery. Rats were anaesthetized with isoflurane (2%), and the carotid artery was cannulated for blood pressure recording. Arterial blood pressure was measured with a microtip catheter pressure transducer (TP-400T; Nihon Kohden, Ltd., Tokyo, Japan) connected to a carrier amplifier (AP-601G; Nihon Koden, Tokyo, Japan). The left carotid artery was ligated for 30 s to observe the baroreflex responses.

2.7. Statistical analysis

Results are expressed as means ± standard error (S.E.). Statistical significance was determined by analysis of variance (ANOVA) followed by the Dunnett test; p values <0.05 were considered to indicate significant differences.

3. Results

3.1. Expression profile of orexin system

To investigate the influence of overexpressed orexin/ataxin-3 on the sympathetic nervous system, we performed RT-PCR analysis of orexin and orexin receptors in the SCG (Fig. 1A). The orexin/ataxin-3 transgenic rat showed marginal expression of orexin, while wild-type rats showed expression of orexin. Wild-type rats showed low expression of OX1R and OX2R. In contrast, the orexin/ataxin-3 transgenic rat showed increased expression of the two receptors (143 ± 14 and 277 ± 21%, OX1R and OX2R, respectively, $n = 6$ for each gene; Fig. 1A, Supplemental data Fig. 1A), suggesting compensatory increase of two receptors. Expression of β-actin was examined as a control. We also evaluated expression of orexin and its receptors in the heart as a target of sympathetic nervous system. There was no significant difference in the expression of OX1R in the heart, while expression of OX2R was increased (153 ± 19%, $n = 6$; Supplemental Fig 1A). The mRNA expression levels of the β1 and β2 adrenergic receptors in the hearts of wild-type and OXTg rats were also not significantly different.

3.2. Western blot analysis

To further investigate the influence of *orexin/ataxin-3* transgene overexpression on the orexin system at the protein level, we analyzed prepro-orexin, OX1R, and OX2R in the SCG of wild-type and OXTg rats by immunoblotting (Fig. 1B). Western blot analysis confirmed the aforementioned expression profiles (Fig. 1A). GAPDH was used for control. The wild-type rat showed expression of orexin in the SCG, whereas OXTg showed little expression of orexin, confirming minimal orexin expression. Expression of OX1R and OX2R was confirmed in wild-type animals, and increased expression of

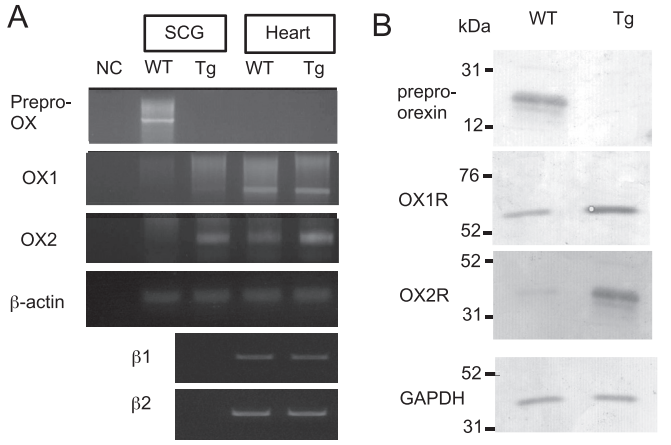

Fig. 1. Expression of prepro-orexin and orexin receptors. (A) RT-PCR analysis of superior cervical ganglion (SCG) in wild-type and *orexin/ataxin-3* transgene over-expressing (OXTg) rats. Identification of prepro-orexin, orexin receptors (OX1R and OX2R), sympathetic β-adrenergic receptor (β1 and β2)-specific transcripts in the SCG or heart (NC, negative control without cDNA; WT, wild-type rats; Tg, *orexin/ataxin-3*-transgenic rats). Expression of β-actin was evaluated as a control. (B) Western blot analysis of the SCG from WT and OXTg rats. Representative immunoblots from WT and OXTg mice analyzed for subunit expression of prepro-orexin, OX1R and OX2R or glyceraldehyde dehydrogenase (GAPDH), as indicated. Significantly decreased prepro-orexin protein levels were observed in OXTg rats along with increased protein levels of OX1R and OX2R. GAPDH was used as control.

two receptors in OXTg was also confirmed (171 ± 15 and 180 ± 9% for OX1R and OX2R, respectively, $n = 6$ for each receptor). Both anti-GAPDH antibody binding and Ponceau-S staining (data not shown) confirmed comparable loading of proteins.

3.3. ECG analysis

We assessed ECG in the wild-type and OXTg rats. ECG of the OXTg rat revealed a regular pattern indicative of physiological pace making and excitation propagation (Fig. 2A). ECG analysis using the "average view" program revealed a normal ECG waveform pattern in the OXTg rats (Supplemental data Fig. 2 and Table 1). There was no significant genotype difference in the ECG intervals, such as R–R intervals, the QRS duration, or QT time (Supplemental Table 1). There were no significant differences in the R–R interval or heart rate at baseline between the wild-type and OXTg rats (Fig. 2B).

To assess sympathetic nerve function in the OXTg rats, we examined the carotid baroreflex function. Wild-type rats exhibited a significant increase in HR, whereas a limited response was observed in the OXTg rats (Fig. 2B). Intraperitoneal administration of propranolol (1.0 mg/kg, i.p.) resulted in a prolonged R–R interval in the wild-type rats, whereas a decreased response was observed in the OXTg rats (representative traces in Fig 2A, statistical data in Fig. 2B). The effects of parasympathetic blockage were also analyzed using injections of atropine (0.5 mg/kg, i.p.), a typical muscarinic receptor antagonist. Atropine significantly shortened the R–R interval in wild-type and OXTg rats (Fig. 2B).

We next analyzed HRV, which was measured as the standard deviation of the interbeat interval. Mean R–R intervals fluctuated more in the OXTg than in the wild-type rats (Fig. 2C), indicating enhanced heart rate variability in OXTg rats. Injection of atropine (0.5 mg/kg, i.p.) shortened the R–R interval, whereas injection of propranolol (1.0 mg/kg, i.p.) lengthened the R–R interval. Statistical analysis revealed increased SDNN in OXTg rats compared with baseline. Atropine injection reduced SDNN in both rat types, with a significant difference between wild-type and OXTg rats.

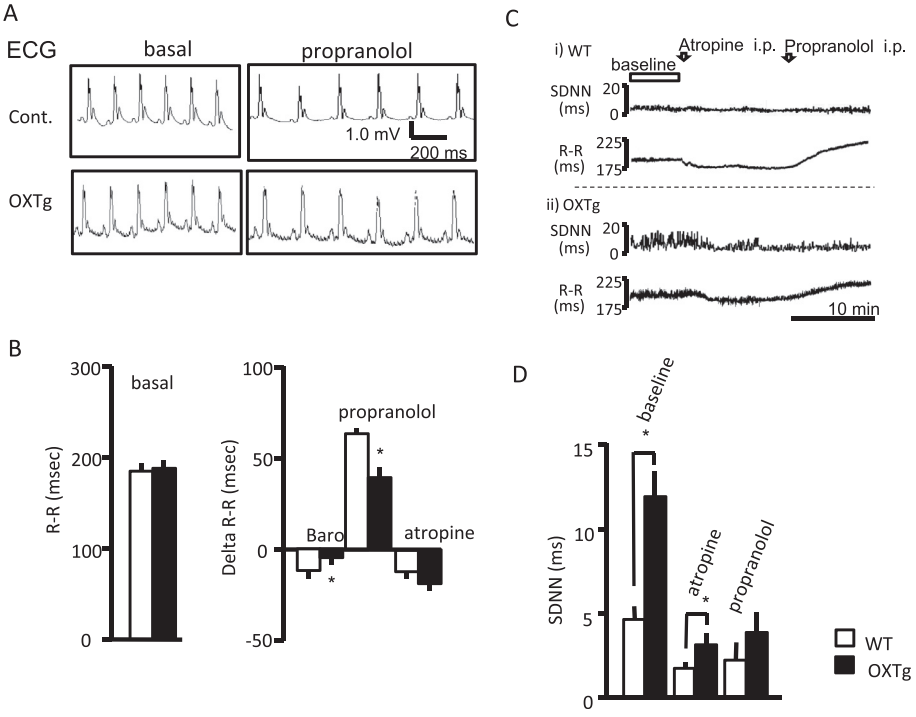

Fig. 2. Decreased sympathetic R–R regulation in the OXTg rat. (A) Representative ECG recordings at the basal state (left panels) and 10 min after an intraperitoneal propranolol injection (right panels). Time-dependent changes in the R–R interval and heart rate in wild-type rats (upper panels). (B) Statistical analysis of the R–R interval (left panel), baroreflex (Baro) and pharmacological responses to propranolol and atropine. Data are expressed as the difference between baseline and each manipulation. Open bar: wild-type; closed bar: OXTg. The physiological and pharmacological manipulations are identified above the bars. *$P < 0.05$ indicates a significant difference between wild-type and Tg rats. Each group contained at least seven rats. **Deteriorated R–R interval regulation**. (C) Representative traces of calculated SDNN (standard deviation of the R–R interval) and R–R interval changes in wild-type (i) and OXTg (ii) rats. Pharmacological manipulation (intraperitoneal injection; i.p., arrow) with atropine and propranolol are indicated. (D) Statistical analysis of SDNN at the basal state and pharmacological effect of atropine and propranolol in the wild-type (open bar) and OXTg (closed bar) rats. *$P < 0.05$ indicates a significant difference between wild-type and Tg rats. Each group contained at least seven rats.

Propranolol injection resulted in no significant differences between wild and OXTg rats in SDNN, suggesting complete blockade of sympathetic control.

We further analyzed HRV in the wild-type and OXTg rats. Fig. 3A shows typical results of beat-to-beat dynamics with Poincare plots (RR_n vs. RR_{n+1}). Obviously, OXTg rats showed fluctuation changes in beat-to-beat dynamics. In the frequency domain analysis, LF (low frequency; 0.2–0.75 Hz) and HF (high frequency; 0.75–2.5 Hz) components were resolved in power spectral density (Fig. 3B). LF components were not significantly different between groups (Fig 3Ci). Statistical analysis of HF components revealed a significant increase in OXTg rats (Fig. 3Cii). As expected from the calculation of the HF component, OXTg rats showed a significantly lower LF/HF ratio than did the wild-type rats (Fig. 3Ciii), suggesting decreased sympathetic tone.

3.4. Cardiachypotrophy

Persistent ablation of orexin had a marked effect on cardiac structure and function. Gross examination revealed that the hearts of 12-week-old OXTg rats were smaller than those of wild-type control rats (Fig. 4A). The heart-to-body weight ratios in 12-week-old wild-type and OXTg rats were also slightly but significantly different (wild-type: 4.2 ± 0.11, $n = 8$; OXTg: 3.95 ± 0.06, $n = 10$; $^*p < 0.05$ vs. wild-type rats, Fig. 4B). Histological analysis (HE staining) of hearts from 12-week-old wild-type and OXTg rats confirmed the hypotrophic effect of orexin ablation (Fig. 4C and D). To better understand the cardiomyopathic changes in the hearts, cross sectional area (CSA) was determined by planimetry (Fig. 4D). As expected, OXTg rats showed significantly smaller CSA, suggesting hypotrophic cardiac changes in OXTg rats.

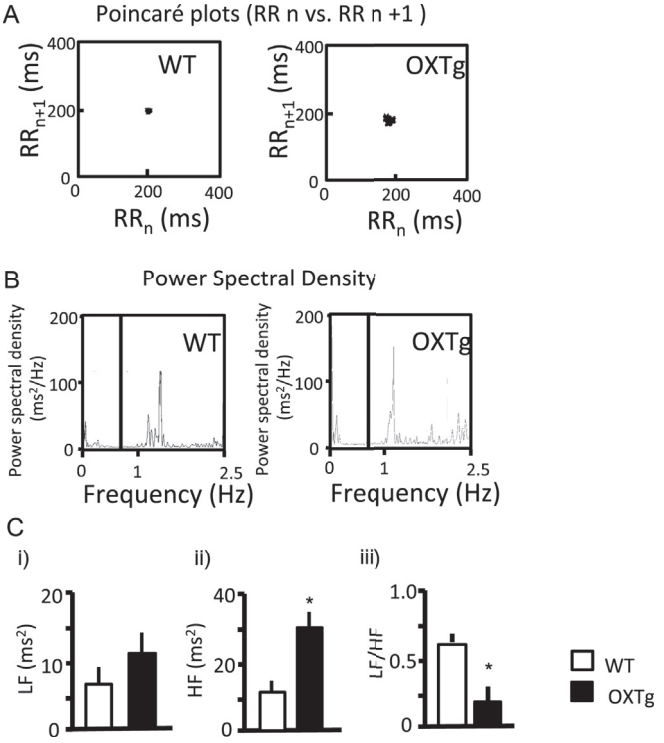

Fig. 3. Modified heart rate variability (HRV). (A) Representative HRV analysis of wild-type (WT: left panel) and OXTg (Tg: right panel) rats. Poincare plots (RRn vs. RRn+1) in which consecutive pairs of R–R intervals during the control period are graphed with the nth+1 R–R interval plotted against the nth R–R period. Note the marked scattering in OXTg rats. (B) Representative power spectral densities of wild-type (WT: left panel) and OXTg (OXTg: right panel) rats. OXTg showed apparently unstable R–R intervals. (C) Statistical comparison of LF (i), HF (ii), and LF/HF ratio (iii). $^*P < 0.05$ indicates a significant difference between wild-type and Tg rats. Each group contained at least seven rats.

3.5. Blood pressure changes

Typical blood pressure traces of wild-type and OXTg rats are shown (Fig. 4E, upper and lower panels, wild-type and OXTg rats, respectively). OXTg rats showed decreased systolic pressure (wild-type: 124.3 ± 3.0, $n = 10$; OXTg: 112.6 ± 2.6*, $n = 10$; $^*p < 0.05$ vs. wild-type rats), whereas diastolic blood pressure (wild-type: 87.7 ± 3.0, $n = 10$; OXTg: 82.2 ± 2.8, $n = 10$) was preserved (Fig. 4F, DBP). OXTg rats showed decreased pulse pressure (difference between systolic and diastolic blood pressure), which was due to decreased systolic pressure in the OXTg rats.

3.6. Pharmacological responses

Because we found a significantly decreased baroreflex response in the aforementioned ECG analysis (Fig. 2B), we next analyzed the baroreflex in blood pressure measurement. The baroreflex function is dependent on carotid baroreceptors in the carotid sinus, which detect changes in aortic blood pressure and regulate blood pressure by activating sympathetic or parasympathetic nerve impulses from the nucleus of the tractus solitarius in the brain stem. Occlusion of the bilateral carotid arteries (30 s) resulted in a significant increase in mean blood pressure in the wild-type rats (3.8 ± 0.4, $n = 10$), whereas a limited response was observed in OXTg rats (2.9 ± 0.2*, $n = 10$; $^*p < 0.05$ vs. wild-type rats, Fig. 4G). The administration of propranolol (1.0 mg/kg, i.p.) resulted in decreased blood pressure in the wild-type rats, whereas a limited decrease was observed in the OXTg rats (wild-type: -6.3 ± 0.4, $n = 10$; OXTg: -4.8 ± 0.5, $n = 10$; $^*p < 0.05$ vs. wild-type rats, Fig. 4G).

4. Discussion

In the present study, we examined the importance of the orexin system in regulation of the autonomic nerve system in the *orexin/ataxin-3* transgenic rat (OXTg), which has a minimal number of orexin neurons. RT-PCR analysis showed expression of orexin and orexin receptor (OX1R) in the SCG of wild-type rats, but expression of the other receptor (OX2R) was low. In the OXTg rat, expression of prepro-orexin was negligible, and expression of orexin receptors (OX1R and OX2R) was increased. Western blot and immunohistochemical analysis confirmed the increased expression of OX1R and OX2R, although expression of orexin was undetectable, suggesting a compensatory increase in both receptors.

ECG and blood pressure recording indicated that OXTg rats showed decreased baroreflex and responsiveness to β-adrenergic blockers. The animals also showed deteriorated R–R interval regulation, indicating involvement of the orexin system in sympathetic nerve regulation. Echocardiogram recording also showed decreased responsiveness to β-adrenergic blocker in the OXTg rats. Histological examination revealed hypotrophic changes in the heart, suggesting involvement of the orexin system in cardiac development.

The OXTg rat has a small number of orexin neurons, as often happens with this type of transgenic approach, although RT-PCR and Western blot analysis confirmed the negligible expression of orexin in the SCG. Nevertheless, a somatic orexin gene exists in the transgenic rat, and further analysis will be needed.

Centrally administered orexins have cardiovascular effects including elevation of blood pressure and heart rate [12]. In a study by Shirasaka et al., an orexin-induced increase in sympathetic nerve outflow led to an increase in plasma norepinephrine,

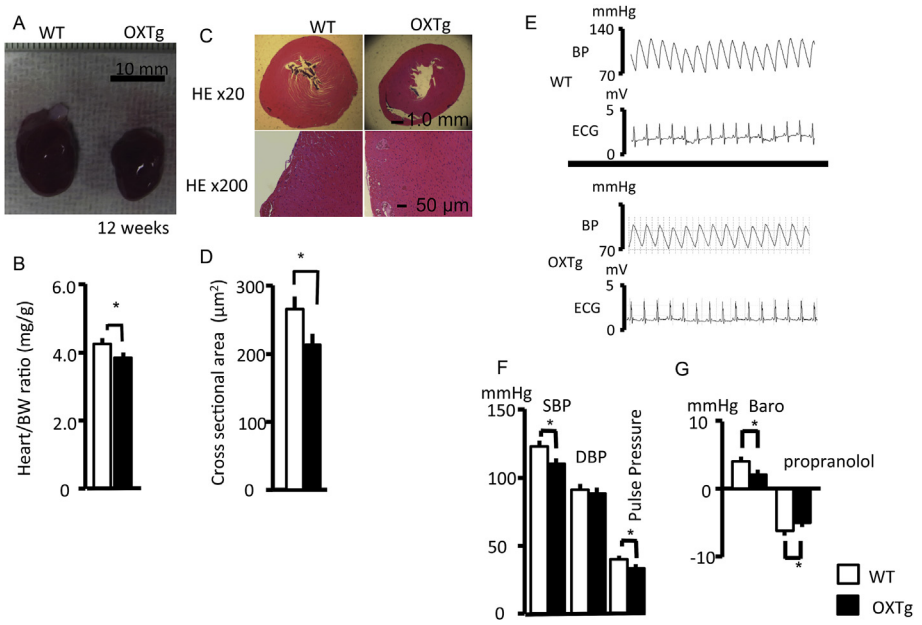

Fig. 4. Hypotrophic heart in OXTg rat. (A) Representative images of hearts in WT and OXTg rats at 12 weeks; scale bar = 10 μm. (B) Heart-to-body weight ratios (mg/g). Data are expressed as the means ± S.E. of at least seven animals. $^{*}P < 0.05$ vs. wild-type control. (C) Histological analysis of hearts from 12-week-old WT and OXTg rats. Hearts were sectioned transversely and stained with HE. (D) Cross-sectional area of 12-week-old WT (open bar) and OXTg (closed bar) rats. Cross-sectional area (CSA) measurements were obtained from a minimum of 200 cardiomyocytes from wild-type and OXTg rats. Four images from non-overlapping regions of each tissue cross section stained with HE were used for CSA measurements. Mean fiber CSA of respective fiber types was determined by planimetry. $^{*}P < 0.05$ vs. wild-type control. **Decreased systolic blood pressure (SBP) and pulse pressure in OXTg rats**. (E) Representative blood pressure and ECG changes in the wild-type (WT; upper panel) and OXTg (OXTg; lower panels) rats. (F) Summarized results of systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure. $^{*}P < 0.05$ indicates a significant difference between wild-type and Tg rats. Each group contained at least seven rats. (G) Statistical analysis of the pharmacological baroreflex (Baro) responses to propranolol and atropine. Data are expressed as the difference between baseline and each manipulation. Open bar, WT; closed bar, OXTg. The physiological and pharmacological manipulations are identified above the bars.

producing cardiovascular responses. Additionally, they showed elevated circulating levels of epinephrine, as well as norepinephrine, after injections of a high dose of orexin-A [12]. In a preliminary study, OXTg rats showed no significant changes in plasma catecholamine (norepinephrine, epinephrine, and dopamine) levels compared with wild-type rats (Kushikata et al., personal communication). This discrepancy may be due to compensation during development. Although there are some inconsistent results, our present results showing decreased blood pressure and sympathetic tone in OXTg rats were consistent with earlier observations. Using the same transgenic rat model, Schwimmer et al., reported decreased blood pressure in accordance with our present results [13]. This was supported by data from Van den Top et al., who reported increased excitability in rat sympathetic neurons by orexin [14]. They examined response to orexin in sympathetic preganglionic neuron (SPN) of the spinal cord, which are innervated by dense orexinergic projections. Taken together, these findings suggest that the orexin system contributes to the control of sympathetic tone.

Interestingly, the SCG showed orexin expression, strongly suggesting expression of orexin neurons in this region. Furthermore, the SCG has two types of orexin receptors (OX1R and OX2R, Fig 1). Considering the increased expression of OX1R and OX2R, the present results suggest the existence of an orexin system in the SCG with feedback regulation.

The presence of autonomic nervous system changes in narcolepsy–cataplexy (NC) has been reported previously. Erectile dysfunction [15], reduced pupillary oscillations [16], and cardiovascular reactivity dysfunctions [17], which suggest abnormal central modulation of autonomic control, have been reported in human patients. Donald et al. reported that catalepsy was associated with co-activation of sympathetic and parasympathetic autonomic systems in narcolepsy–cataplexy patients [18]. Nevertheless, further study will be needed to clarify the relation between orexin and the autonomic nerve system.

In conclusion, the OXTg rat showed decreased sympathetic nerve tone, strongly suggesting an excitatory effect of orexin on the sympathetic nervous system. Future clarification of the role of orexins in sympathetic control will require spatially restricted pharmacological manipulations to localize the site of orexin actions, gene-targeted receptor manipulation, such as OX1R- or OX2R-deficient mice, or selective antagonists to distinguish their contribution.

Conflict of interest

None.

Acknowledgements

The authors declare that they have no conflict of interest. The authors thank Dr. Koichi Wakabayashi and Fumiaki Mori for their assistance. We also thank Dr. Masashi Yanagisawa.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.157.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.157.

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