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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