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Original Article

# Involvement of orexin in lipid accumulation in the liver

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ABSTRACT

*Objectives:* Orexin, a hypothalamic neuropeptide, is involved in energy homeostasis and regulates motivated behaviors, including feeding and the awake-sleep cycle. Orexin-knockout (OX-KO) mice exhibit greater weight gain than wild-type (WT) mice, despite similar food intake, suggesting that OX-KO mice may have an altered metabolism for weight gain. However, the actual effects of orexin on the metabolism of mice remain unclear.

*Methods:* We compared the lipid metabolism of OX-KO and WT mice, with a focus on lipid metabolism in the liver.

*Results:* The livers of OX-KO mice were significantly larger than those of WT mice and were yellowish in appearance. Oil red O staining revealed that lipid accumulation in the livers of OX-KO mice was higher than that in WT mice. Hepatic triglyceride content in OX-KO mice was higher than that in WT mice. The total cholesterol level in the peripheral blood was higher in OX-KO mice than in WT mice, although no significant differences in free fatty acid and serum triglyceride levels were observed. In comparison with WT mice, OX-KO mice showed higher transcript levels of hepatic lipogenic genes encoding sterol regulatory element binding protein 1 and *Cd36* and lower transcript levels of genes encoding fatty acid transporter protein 2/5, peroxisomal proliferator-activated receptor α, STAT-induced suppressor of cytokine signaling 3, and hormone-sensitive lipase. Moreover, tumor necrosis factor *(TNF)* $\alpha$ mRNA levels in the liver were significantly higher in OX-KO mice than in WT mice.

*Conclusions:* Orexin likely contributes to the regulation of lipid metabolism in the liver.



## 1. Introduction

Orexin neurons are located in the hypothalamus and are widely distributed throughout the central nervous system [1–5]. Orexin has been shown to be involved in the maintenance of wakefulness, physical activity, reward-seeking, feeding, and locomotor activity [6–10]. The loss of hypothalamic orexin neurons results in the chronic sleep disorder narcolepsy [11]. Narcoleptic subjects with undetectable levels of orexin in the cerebrospinal fluid (CSF) have been reported to often show a higher body mass index than narcoleptic subjects with detectable CSF orexin levels [12]. In a previous study, orexin-knockout (OX-KO) mice and orexin neuron-ablated mice (orexin/ataxin-3 transgenic mice) showed phenotypes similar to those observed in human narcolepsy [13,14]. Both Ox-KO mice and orexin neuron-ablated mice are significantly heavier than WT mice; however, the food intake of these narcoleptic mice is not higher than that of WT mice [14,15]. Moreover, in a preliminary study, we found that the livers of OX-KO mice were yellowish in appearance, typical of fatty liver, and larger than those of WT mice. It is possible that the lipid metabolism in the liver of OX-KO mice may be altered in comparison with that in WT mice; however, it is not clear whether orexin affects lipid metabolism in the liver.

*Abbreviations:* Srebp1, Sterol regulatory element binding protein 1; Cd36, Cluster of differentiation 36; Ppar, Peroxisomal proliferator-activated receptor; Ucp2, Uncoupling protein 2; Socs3, STAT-induced suppressor of cytokine signaling 3; Fatp2/5, Fatty acid transporter protein 2/5; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; Tnfα, Tumor necrosis factor α; Cpt1, Carnitine palmitoyltransferase 1; Ucp2, Uncoupling protein 2; Hsl, Hormone-sensitive lipase; Fas, Fatty acid synthase; Scd1, Stearoyl-CoA desaturase-1; Pepck, Phosphoenolpyruvate carboxykinase; PCR, Polymerase chain reaction; OX-KO, Orexin-knockout; WT, Wild type; CSF, Cerebrospinal fluid; SPF, Specific pathogen-free

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In the present study, to investigate whether lipid metabolism in OX-KO mice is altered in comparison with that in WT mice, we examined the expression of genes associated with lipid metabolism in the liver. The results suggest that orexin deficiency may induce hepatic lipid accumulation via increased expression of sterol regulatory element binding protein 1 (*Srebp1*), decreased expression of peroxisomal proliferator-activated receptor α (*Pparα*), and increased expression of cluster of differentiation 36 (*Cd36*), and this lipid accumulation may be involved in the induction of inflammation in the liver of OX-KO mice.

## 2. Materials and methods

### 2.1. Animals

OX-KO mice with a mixed C57BL/6J-129/SvEv background that completely lacked the prepro-orexin gene were generated using previously described standard methods [13]. Heterozygous mice were mated to obtain null (-/-) mutants and wild-type (WT; +/+) littermates. Male OX-KO mice that were 12–27 weeks old and their WT littermates were used in this study. All OX-KO mice and their WT littermates were reared in a specific pathogen-free (SPF) room and were given free access to distilled water and food, which their mothers were also fed on. Body and liver weights were measured as the final body weight at the time of liver harvest. Mice were genotyped at weaning by polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tail using a REDExtract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO, USA). All mice were housed in SPF rooms and given free access to distilled water and pelleted food containing 20% protein and 9% fat (5058 PicoLab Mouse Diet 20; Japan SLC Inc., Hamamatsu, Japan) under a 12-h light/dark cycle (lights on from 08:00 to 20:00) and controlled temperature conditions (23 ± 1 °C). The animal experiments complied with the ARRIVE guidelines and were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### 2.2. Histology

Mice were deeply anesthetized with an overdose of ketamine hydrochloride (intraperitoneal [i.p.] injection, KETALAR®; DAIICHI SANKYO, Tokyo, Japan) and xylazine hydrochloride (i.p. injection, Sigma-Aldrich, St. Louis, MO, USA), and their livers were collected fresh and visualized macroscopically (Fig. 1A). Additional mice were deeply anaesthetized with an overdose of ketamine hydrochloride and xylazine hydrochloride, and perfused with saline followed by 4% paraformaldehyde through the left ventricle. Livers were embedded in optical cutting temperature compound (O.C.T. Compound; Sakura Finetek, Torrance, CA) and then frozen. Sections with a thickness of 10 μm were obtained from the frozen tissues embedded in O.C.T. Compound by using a cryostat (Leica CM3050S, Wetzlar, Germany) and stained with Oil Red O by Biopathology Institute Co., Ltd. (Oita, Japan). Tissue sections were mounted on glass slides, and images were obtained using a microscope (models BZ-9000 and BZ-X710; Keyence, Osaka, Japan).

### 2.3. Measurement of cholesterol, free fatty acid, and triglyceride levels

Mice, which were fed *ad libitum*, were anesthetized with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.), and their sera were collected. Serum cholesterol, free fatty acid, and triglyceride levels were determined by the Ultra Violet-End (UV-End) method using cholesterol dehydrogenase, the enzymatic method, and the glycerol-3-phosphate oxidase method, respectively, by SRL Inc. (Tokyo, Japan). Hepatic triglyceride content was determined by Skylight Biotech, Inc. (Akita, Japan) using Cholestest TG (Sekisui Medical, Co., Ltd., Tokyo, Japan).

### 2.4. Quantitative real-time PCR

Mouse livers were homogenized with TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was obtained using TRIzol. First-stand cDNA was synthesized for PCR using Superscript III (Invitrogen) and subjected to amplification with Taq polymerase (Sigma-Aldrich). The mRNA expression levels of *Srebp1*, *Pparα*, *Cd36*, *Fatp2*, *Fatp5*, *Socs3*, *Pparγ*, *Ucp2*, *Tnfα*, *Hsl*, *Cpt1*, *Fas*, *Scd1*, Pepck, and *Gapdh* were quantified using a TaqMan Real-time PCR system (Thermo Fisher Scientific Inc. Waltham, MA, U.S.A.) with mouse *Srebp1* (Assay ID: Mm01138344_m1), mouse *Pparα* (Assay ID: Mm00440939_m1), mouse *Cd36* (Assay ID: Mm01135198_m1), mouse *Fatp2* (Assay ID: Mm00449517_m1), mouse *Fatp5* (Assay ID: Mm00447768_m1), mouse *Socs3* (Assay ID: Mm01249143_g1), mouse *Pparγ* (Assay ID: Mm00440945_m1), mouse *Ucp2* (Assay ID: Mm00627599_m1), mouse *Tnfα* (Assay ID: Mm00440945_m1), mouse *Hsl* (Assay ID: Mm00495359_m1), mouse *Ctp2* (Assay ID: Mm01231183_m1), mouse *Fas* (Assay ID: Mm01253292_m1), mouse *Scd1* (Assay ID: Mm00772290_m1), mouse Pepck (Assay ID: Mm00440637_g1), and mouse *Gapdh* (Assay ID: Mm99999915_g1) probes in accordance with the manufacturer's instructions.

### 2.5. Statistical analyses

Intergroup differences in data were analyzed using Student's *t*-test and Mann-Whitney *U* test, and all data are presented as the mean ± SD values; $p < 0.05$ was considered significant. Statistical analyses were conducted with SPSS 22.0J (IBM Japan Inc., Tokyo, Japan).

## 3. Results

### 3.1. Appearance of livers in OX-KO mice and changes in cholesterol levels in the peripheral blood of OX-KO mice

We previously found that the intake of a pelleted food containing 20% protein and 9% fat did not differ between WT and OX-KO mice; however, OX-KO mice gained weight more rapidly than WT mice [15]. In the present study, mice were fed under the same conditions as previously described [15], and OX-KO mice gained more weight than WT mice (WT, 37.2 ± 7.7 g, n = 25; KO, 43.3 ± 5.8 g, n = 23; p < 0.01; Fig. 1B, left panel), and the liver weight of OX-KO mice was higher than that of WT mice (WT, 1.85 ± 0.74 g, n = 25; KO, 2.48 ± 0.90 g, n = 23; p < 0.01; Fig. 1B, middle panel). Since obese patients often show liver diseases such as lipid accumulation in the liver [16], we examined whether the histological characteristics of the liver differed between WT mice and OX-KO mice. Livers from OX-KO mice were yellowish in appearance, typical of fatty liver, and appeared to be enlarged relative to those of WT mice (Fig. 1A). The liver-to-body weight ratio was slightly but significantly higher in OX-KO mice (Fig. 1B; WT, 4.88 ± 1.06%, n = 25; KO, 5.62 ± 1.47%, n = 23; p < 0.05 Fig. 1B, right panel). Moreover, OX-KO mice showed lipid accumulation in the liver with Oil Red O staining, which detects the accumulation of lipids (Fig. 1C). We next compared the changes in the triglyceride content in the liver. The hepatic triglyceride content in OX-KO mice was significantly higher than that in WT mice (Fig. 1D). Because several studies have also assessed serum lipid constituents, including cholesterol, free fatty acids, and triglycerides, to examine

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