Hypothalamic orexin prevents non-alcoholic steatohepatitis and hepatocellular carcinoma in obesity

Graphical abstract

Highlights

- Orexin deficiency exacerbates diet-induced obesity and hepatic steatosis
- Orexin deficiency triggers the progression from steatosis to NASH and HCC
- Orexin improves hepatic ER homeostasis through the autonomic nervous system
- Daily administration with orexin prevents NASH-causing inflammatory events

Authors

Hiroshi Tsuneki, Takahiro Maeda, Shinjiro Takata, ..., Akihiro Yamanaka, Tsutomu Wada, Toshiyasu Sasaoka

Correspondence

htsuneki@pha.u-toyama.ac.jp (H.T.), tsasaoka@pha.u-toyama.ac.jp (T.S.)

In brief

Tsuneki et al. reveal that central actions of orexin are required for preventing NASH and hepatocellular carcinoma (HCC) under diet-induced obese conditions. In this process, orexin attenuates hepatic endoplasmic reticulum (ER) stress-associated inflammation via the autonomic nervous system in addition to causing the anti-obese effect via promotion of physical activity.
Non-alcoholic steatohepatitis (NASH) occasionally occurs under obesity; however, factors modulating the natural history of fatty liver disease remain unknown. Since hypothalamic orexin that regulates physical activity and autonomic balance prevents obesity, we investigate its role in NASH development. Male orexin-deficient mice fed a high-fat diet (HFD) show severe obesity and progression of NASH with fibrosis in the liver. Hepatic fibrosis also develops in ovariectomized orexin-deficient females fed an HFD but not ovariectomized wild-type controls. Moreover, long-term HFD feeding causes hepatocellular carcinoma (HCC) in orexin-deficient mice. Intracerebroventricular injection of orexin A or pharmacogenetic activation of orexin neurons acutely activates hepatic mTOR-sXbp1 pathway to prevent endoplasmic reticulum (ER) stress, a NASH-causing factor. Daily supplementation of orexin A attenuates hepatic ER stress and inflammation in orexin-deficient mice fed an HFD, and autonomic ganglionic blocker suppresses the orexin actions. These results suggest that hypothalamic orexin is an essential factor for preventing NASH and associated HCC under obesity.
Figure 1. Long-term HFD feeding causes NASH with fibrosis in male Ox⁻/⁻ mice

(A–J) Male WT and Ox⁻/⁻ mice were fed an HFD or an NCD for 16 weeks.

(A) Body weight.

(B) Tissue weight.

(C) Liver.

(D) H&E.

(E) NAS (16 W).

(F) Fibrosis (16 W).

(G) Triglyceride.

(H) AST.

(I) mRNA levels (fold).

(J) Col1a1.

(K) H&E.

(L) NAS (24 W).

(M) Fibrosis (24 W).

(legend continued on next page)
physical activity (SPA) and associated non-exercise activity thermogenesis (NEAT) is known to be required for preventing obesity. For instance, activation of orexin neuron counteracted a reduction of SPA and NEAT in HFD-fed obese mice (Sunney et al., 2017), and orexin-overexpressing mice were resistant to diet-induced obesity (Funato et al., 2009). Orexin knockout (Ox−/−) mice exhibited severe obesity under HFD-fed conditions despite no change in food intake (Tsuneki et al., 2008; Kakizaki et al., 2019). Furthermore, regulation of the autonomic nervous system by orexin was necessary for maintaining peripheral insulin sensitivity (Tsuneki et al., 2016b). Ox−/− mice fed a normal chow diet (NCD) exhibited hepatic insulin resistance during aging (Tsuneki et al., 2015) and under a psychological stress condition (Tsuneki et al., 2013). Thus, Ox−/− mice fed an HFD could be utilized as a model that combines overnutrition, physical inactivity, and autonomic imbalance. However, the influence of long-term HFD feeding on the development of NASH and HCC in Ox−/− mice has remained to be examined.

Women have a lower risk of NAFLD than men, but fatty liver diseases occur at a higher rate in the postmenopausal state (Lonardo et al., 2019). Previous studies with animal models of estrogen deficiency have suggested that estrogen plays a fundamental role in the prevention of obesity, NAFLD, and NASH in females (DiStefano, 2020; Ishikawa et al., 2020). It is of note that estradiol supplementation increased the numbers of activated orexin neurons in the hypothalamus of ovariectomized rats (Deuveilhier et al., 2008). Expression of orexin in the hypothalamus is sexually dimorphic, namely, it is higher in females (Johren, 2018). These raise a possibility that estrogen might exert metabolically beneficial effects in an orexin-dependent manner in females. However, the functional relationship between orexin and estrogen against NAFLD progression in the postmenopausal state remains to be investigated.

To clarify the physiological significance of the orexin system for maintaining metabolic health, the present study examined whether long-term HFD feeding causes NASH and HCC in Ox−/− mice. In addition, the role of orexin in NASH development in the postmenopausal state was investigated by using ovariec-
tomized Ox−/− mice fed an HFD. To reveal the protective mechanism by orexin, the effects of intracerebroventricular (i.c.v.) injection of orexin A and pharmacogenetic activation of orexin neurons on hepatic ER homeostasis were analyzed. Furthermore, we examined whether daily orexin A supplementation can prevent NASH-related ER stress and inflammation in the liver of Ox−/− mice fed an HFD. Thus, this study focused on the brain system regulating physical activity and autonomic balance as the fundamental mechanism to prevent/halt NASH under diet-induce obese conditions.

RESULTS

Progression of NASH in male Ox−/− mice fed an HFD

Male Ox−/− mice showed severe obesity (Figure 1A) and reduced locomotor activity (Figure 1B) during exposure to HFD compared with wild-type (WT) male mice. Energy expenditure and food intake were comparable between WT and Ox−/− mice (Figures S1A and S1B). After 16 weeks of HFD feeding, Ox−/− mice showed increased weights of the inguinal white adipose tissue (iWAT) and liver in association with reduced weight of the epididymal WAT (eWAT) compared with WT mice (Figure 1C). In hematoxylin and eosin (H&E) staining, marked hepatic steatosis was detected in Ox−/− mice (Figure 1D; Table S1). Ox−/−, but not WT, mice showed increased total NAFLD activity score (NAS), higher than a threshold for the diagnosis of NASH (NAS ≥ 5; Sheka et al., 2020) (Figure 1E). Sirius red staining showed no fibrotic changes at this time point (Figures 1D and 1F). Hepatic triglyceride content (Figure 1G), serum levels of AST, ALT, and fibrosis areas (F) in the liver. Scale bars: 200 μm.

(G) Hepatic triglyceride content.
(H) Serum levels of AST and ALT.
(I) The expression levels of hepatic inflammation markers.
(J) The levels of hepatic fibrosis markers in mice fed an HFD for 16 weeks. n = 4–5/group.
(K–M) Male WT and Ox−/− mice were fed an HFD or an NCD for 24 weeks.
(N) The NAS.
(M) Fibrosis areas. n = 5–6/group.

* p < 0.05 (WT-NCD versus Ox−/−-NCD) determined by ANOVA with post hoc test.
* p < 0.05 (WT-NCD versus WT-HFD) determined by ANOVA with post hoc test.
* p < 0.05 (WT-NCD versus Ox−/−-HFD) determined by ANOVA with post hoc test.
* p < 0.05 (Ox−/−-NCD versus WT-HFD) determined by ANOVA with post hoc test.
* p < 0.05 (Ox−/−-NCD versus Ox−/−-HFD) determined by ANOVA with post hoc test.
* p < 0.05 determined by Student’s t test. Data are represented as mean ± SEM. See also Figures S1, S2, and S4 and Tables S1 and S2.
Figure 2. Orexin deficiency causes NASH with fibrosis in ovariectomized mice fed HFD or HFFD

(A–F) Sham-operated WT, ovariectomized (OVX)-WT, sham-operated Ox−/−, and OVX-Ox−/− mice were fed an HFD for 20 weeks.

(A) Body weight.

(B) Weights of the iWAT, gWAT, and liver.

(C) Hepatic triglyceride (TG) content.

(D) H&E and Sirius red staining.

(E) NAS (HFD).

(F) Fibrosis (HFD).

(G) Body weight.

(H) Weights of the iWAT, gWAT, and liver.

(I) Hepatic triglyceride (TG) content.

(J) H&E and Sirius red staining.

(K) NAS (HFD).

(L) Fibrosis (HFD).

(legend continued on next page)
pathways related to this miRNA profile included endocrine system disorders such as diabetes mellitus and NAFLD (Figure S1C), suggestive of their intermediary role. After 24 weeks of HFD feeding, Ox⁻/⁻ mice exhibited significant increases not only in total and two components (steatosis and ballooning) of the NAS (Figures 1K and 1L; Table S1) but also the fibrosis stages and areas (Figures 1K, 1M, and S1D) compared with WT controls. These results indicate that orexin deficiency caused NASH in male mice fed an HFD.

Concomitantly in adipose tissue, male Ox⁻/⁻ mice fed an HFD for 16 weeks showed marked increases in the levels of biochemical (Tnfa, Ccl2, Tlr4, Emr1, Cd11c, and Cd206 mRNAs) and histological markers (i.e., crown-like structure) of inflammation in the eWAT when compared with WT mice (Figures S1E and S1F). On the other hand, there were no changes in histological profiles and the Ucp1 mRNA levels in the BAT of Ox⁻/⁻ mice (Figures S1G and S1H). Serum levels of non-esterified fatty acid (NEFA), insulin, and leptin were not different between WT and Ox⁻/⁻ mice (Figure S1I).

**Progression of NASH in female Ox⁻/⁻ mice fed HFD**

Female Ox⁻/⁻ mice fed an HFD also showed severe obesity compared with WT females (Figure S2A). After 24 weeks of HFD feeding, weights of the iWAT, gonadal WAT (gWAT), and liver were heavier in Ox⁻/⁻ than WT females (Figure S2B). Hepatic triglyceride content (Figure S2C), the levels of hepatic inflammation markers (Tnfa, Ccl2, and Emr1 mRNAs) (Figure S2D), the levels of hepatic fibrosis markers (Col1a1, Tgfb1, and Acta2 mRNAs) (Figure S2E), and the levels of adipose tissue inflammation markers (Tnfa, Ccl2, Tlr4, Emr1, Cd11c, and Cd206 mRNAs in the gWAT) (Figure S2F) were elevated in female Ox⁻/⁻ mice fed an HFD for 24 weeks when compared with the WT controls. However, neither the NASs nor Sirius red-stained areas increased in the Ox⁻/⁻ females at this time point when compared with the WT levels (Figures S2G–S2I; Table S1), indicating that there were sex differences in the progression rate of hepatic fibrosis between male and female Ox⁻/⁻ mice during HFD feeding.

Since estrogen participates in the prevention of NASH (Kamada et al., 2011), we compared the impacts of orexin and estrogen deficiency on NASH progression in female mice fed an HFD for 20 weeks. At the 20th week, body weights (Figure 2A), weights of the iWAT, gWAT, and liver (Figure 2B), and hepatic triglyceride content (Figure 2C) were increased in sham-operated Ox⁻/⁻ and ovariectomized (OVX)-Ox⁻/⁻ mice to similar extent when compared with their WT controls. Energy expenditure and food intake were comparable between OVX-WT and OVX-Ox⁻/⁻ mice fed an HFD (Figures S3A and S3B). Histological analyses demonstrated that total and all components of the NASs (Figures 2D and 2E; Table S1) and the fibrosis stages and areas (Figures 2D, 2F, and S3C) were increased in the liver of OVX-Ox⁻/⁻ mice, while no apparent abnormalities were observed in sham-operated WT, sham-operated Ox⁻/⁻, and OVX-WT mice. In the gWAT, the levels of inflammation markers (Tnfa, Ccl2, and Emr1 mRNAs) were significantly elevated only in OVX-Ox⁻/⁻ mice among the four groups (Figure S3D). Moreover, we examined the influence of 20-week feeding of high-fat high-fructose diet (HFD) that strongly promotes hepatic insulin resistance and NAFLD (Softic et al., 2020) in the four groups of mice. At the 20th week, no significant differences in body weights (Figure 2G), weights of the iWAT, gWAT, and liver (Figure 2H), and hepatic triglyceride content (Figure 2I) were observed among sham-operated WT, OVX-WT, sham-operated Ox⁻/⁻, and OVX-Ox⁻/⁻ mice when fed an HFD, although they were higher than the levels of naive WT mice fed an NCD. Energy expenditure and food intake were not different at least among four groups fed an HFD (Figures S3E and S3F). Nevertheless, total and two components (inflammation and ballooning) of the NASs (Figures 2J and 2K; Table S1) and the fibrosis stages and areas (Figures 2J, 2L, and S3G) were increased exclusively in the liver of OVX-Ox⁻/⁻ mice. In the gWAT, inflammation markers (Tnfa, Ccl2, and Emr1 mRNAs) were elevated only in OVX-Ox⁻/⁻ mice among the groups tested (Figure S3H). These results indicate that the combination of orexin deficiency and ovariectomy, particularly under a more extremely NAFLD-promoting diet, synergistically caused NASH development in female mice.

**Progression of NASH-associated HCC in Ox⁻/⁻ mice fed HFD**

Next, we investigated whether orexin deficiency causes NASH-driven HCC during long-term exposure to an HFD. After 52 weeks...
Figure 3. Prolonged HFD feeding causes NASH-associated HCC in Ox−/− mice
Male WT and Ox−/− mice were fed an HFD or an NCD for 52 weeks.
(A–C) Diet-induced obesity in Ox−/− mice.
(A) Representative photograph of WT and Ox−/− mice.
(B) Body weight.
(C) Tissue weight.
(D) Triglyceride.
(E) Histological analysis of liver tissue.
(F) NAS score.
(G) Fibrosis area.
(H) Timp1 mRNA levels.
(I) AST and ALT serum levels.
(J) Afp and G6pd mRNA levels.
(K–N) mRNA levels of various genes.

(legend continued on next page)
of HFD feeding, severer obesity was observed in male Ox<sup>+/−</sup> mice compared with the WT controls (Figures 3A and 3B), which could not be fully attributed to the specific tissues tested (Figure 3C) and hepatic triglyceride content (Figure 3D). Yet, Ox<sup>+/−</sup> mice fed an HFD showed marked increases in total and two components (inflammation and ballooning) of the NASs (Figures 3E and 3F, Table S1) and the fibrosis stages and areas in the liver specimens (Figures 3E, 3G, and S1D) in association with an increase in the hepatic expression of Timp1 mRNA, a fibrosis marker (Figure 3H). Moreover, tumor formation occurred exclusively in Ox<sup>+/−</sup> mice fed an HFD (Figure 3I). The overall tumor incidence on the liver surface increased significantly in Ox<sup>+/−</sup> mice (WT mice: 0%, Ox<sup>+/−</sup> mice: 65%, p < 0.05 by Pearson’s chi-square test). The levels of HCC (Afp and G6pd mRNAs) were elevated in the non-tumor region of the liver of Ox<sup>+/−</sup> mice compared with the WT controls (Figure 3J). Serum levels of AST and ALT were also elevated in Ox<sup>+/−</sup> mice (Figure 3K). Ox<sup>+/−</sup> mice also exhibited increased levels of ER stress (Chop mRNA and chronic inflammation markers (Ccl2, Emr1, and Cd11c mRNAs) in the liver (Figures 3L and 3M). In the eWAT, the Ccl2 mRNA levels were comparable between Ox<sup>+/−</sup> and WT mice (Figure 3N). These results indicate that orexin deficiency caused NASH-associated HCC under HFD.

**Early pathological process of NAFLD development in Ox<sup>+/−</sup> mice fed HFD**

To reveal the pathological mechanism of NAFLD development by orexin deficiency, the time course of the development of metabolic abnormalities was investigated in male Ox<sup>+/−</sup> mice during short-term (2- to 8-week) exposure to an HFD. Within 2 weeks of HFD feeding, Ox<sup>+/−</sup> mice showed larger increases in body weights (Figure 4A), glucose intolerance (Figure 4B), and weights of the iWAT, eWAT, and liver (Figure 4C) than WT mice fed an HFD. Ox<sup>+/−</sup> mice exhibited severer insulin resistance at the 4th week (Figure 4D). At the 8th week, weights of the iWAT, eWAT, and liver (Figure 4E) and the expression levels of lipogenesis markers (Fasn and Elovl6 mRNAs) in the liver (Figure 4F) were increased in HFD-fed Ox<sup>+/−</sup> mice when compared with HFD-fed WT mice. Serum levels of NEFA and tumor necrosis factor α (TNF-α) were comparable between WT and Ox<sup>+/−</sup> mice (Figure 4G). Insulin resistance associated with glucose intolerance was continuously observed thereafter (i.e., 9th–12th week; Figures S4A and S4B). Female Ox<sup>+/−</sup> mice fed an HFD also showed glucose intolerance and insulin resistance, although OXV had no significant influences on them (Figures S4C and S4D). These results indicate that orexin deficiency rapidly caused diet-induced obesity and insulin resistance, leading to excess hepatic lipogenesis.

We further compared the influence of homozygous (Ox<sup>+/+</sup>) and heterozygous orexin knockout (Ox<sup>+/−</sup>). When fed an HFD, body weight (Figure S5A), insulin tolerance (Figure S5B), and weights of the iWAT, eWAT, and liver (Figure S5C) were comparable between male WT and Ox<sup>+/−</sup> mice, whereas all these parameters were abnormally increased in male Ox<sup>+/−</sup> mice. Energy expenditure (Figure S5D) and food intake (Figure S5E) were not different among WT, Ox<sup>+/−</sup>, and Ox<sup>+/+</sup> mice. The levels of inflammatory markers (Ccl2, Cd11c, and Tnfa mRNAs) were elevated only in the liver of Ox<sup>+/−</sup> mice fed an HFD for 9 weeks when compared with Ox<sup>+/−</sup> and WT controls (Figure S5F). Oxidative stress levels in the serum and the liver, assessed by thiobarbituric-acid-reactive substance (TBARS) method, were not different among three genotypes (Figure S5G). Thus, heterozygous expression of orexin was sufficient to prevent diet-induced obesity and associated insulin resistance and hepatic inflammation.

**Orexin A administration prevents NASH-causing abnormalities in Ox<sup>+/−</sup> mice fed HFD**

Next, we examined whether treatment with orexin A could also prevent NASH development during HFD feeding. After 8 weeks of HFD feeding, orexin A (0.3 nmol/mouse, i.c.v.) was daily administered for 5 days at the beginning of the dark period (Figure 5A) according to a daily rhythm of endogenous orexin secretion. Body weights (Figure 5B), hepatic triglyceride content (Figure 5C), and the levels of de novo lipogenesis (Fasn and Elovl6 mRNAs) and chronic inflammation markers (Ccl2 and Cd11c mRNAs) in the liver were higher in Ox<sup>+/−</sup> than WT mice treated with saline (Figures 5D and 5E). Supplementation of orexin A reduced these mRNA levels (Figures 5D and 5E) without affecting body weights and hepatic triglyceride content (Figures 5B and 5C) in Ox<sup>+/−</sup> mice. In contrast, supplementation

(C) Weights of the iWAT, eWAT, and liver. n = 5–11/group.

(D–M) NASH/HCC-related abnormalities in the liver.

(E–Q) H&E staining (E, top and middle), Sirius red staining (E, bottom), the NAS (F), and fibrosis areas (G) in the liver tissue. Scale bars: 200 μm. n = 7–11/group.

(H) The expression levels of hepatic fibrosis marker (Timp1 mRNA) in the liver. n = 7–11/group.

(I) Tumorigenesis in the liver of Ox<sup>−/−</sup> mice. Arrow heads indicate the site of tumorigenesis.

(J) The expression levels of HCC markers (Afp and G6pd mRNAs) in the liver. n = 7–11/group.

(K) Serum levels of liver damage markers (AST and ALT). n = 7–11/group.

(L) The expression levels of ER stress marker (Chop mRNA) in the liver. n = 7–11/group.

(M) The expression levels of chronic inflammation markers (Ccl2, Emr1, and Cd11c mRNAs) in the liver. n = 7–11/group.

(N) The Ccl2 mRNA levels in the eWAT. n = 7–8/group.

*p < 0.05 (WT-NCD versus WT-HFD) determined by ANOVA with post hoc test.

*<sup>p</sup> < 0.05 (WT-NCD versus Ox<sup>−/−</sup>-HFD) determined by ANOVA with post hoc test.

*<sup>p</sup> < 0.05 (Ox<sup>−/−</sup>-NCD versus WT-HFD) determined by ANOVA with post hoc test.

*<sup>p</sup> < 0.05 (Ox<sup>−/−</sup>-NCD versus Ox<sup>−/−</sup>-HFD) determined by ANOVA with post hoc test.

<sup>p</sup> < 0.05 determined by Mann-Whitney U test.

<sup>p</sup> < 0.05 determined by Student’s t-test. Data are represented as mean ± SEM. See also Figure S1 and Table S1.
of orexin A did not affect the mRNA levels of chronic inflammation markers (Tnfa, Ccl2, and Cd11c) in the eWAT of Ox⁻/⁻ mice fed an HFD (Figure S6A). Flow cytometric analysis also showed no effect of orexin A supplementation on the levels of M1/M2-macrophage polarization markers (CD11c and CD206) in the eWAT of Ox⁻/⁻ mice (Figure S6B). Thus, supplementation of orexin A mainly improved hepatic inflammation in Ox⁻/⁻ mice fed an HFD.
Central actions of orexin promote adaptive ER response in the liver

ER stress underlies the basic pathophysiological mechanism that leads to NASH progression (Baiceanu et al., 2016), and ER stress is known to be enhanced by refeeding after 24 h fasting in mice (Lee et al., 2011). To explore the mechanism of the anti-NASH effect of orexin A, Ox−/− mice fed an HFD or an NCD for 8 weeks were daily administered either orexin A (0.3 nmol/mouse, i.c.v. at ZT14) or saline, and the effects were analyzed on day 5 under 24 h fasted plus 2 h refed conditions, i.e., the enhanced ER-stress condition (Figure 5F). Hepatic expression levels of ER stress markers (phosphorylation of eukaryotic initiation factor 2x [eIF2x] and expression of CHOP) and chronic inflammation markers (phosphorylation of nuclear factor-κB [NF-κB], c-jun N-terminal kinase [JNK], and p38 mitogen-activated protein kinase [MAPK]) were elevated by HFD feeding, and orexin A administration reduced these levels to control ranges (Figures 5G–5I). Pretreatment with hexamethonium, an autonomic ganglionic blocker, eliminated the reducing effects of orexin A on the phosphorylation of eIF2x and expression of CHOP in the liver (Figures 5J–5L). These results indicate that central actions of orexin prevented hepatic ER stress and chronic inflammation under obese conditions through the autonomic nervous system.

Since sympathetic noradrenergic inputs to the liver has been reported to prime adaptive ER response to anticipated food ingestion through hepatic mTOR-sXbp1 pathway (Brandt et al., 2020), we examined whether specific activation of orexin neurons can acutely promote the defensive response in the fasting state by using orexin-flippase knockin (Orexin-Flp) mice expressing hM3Dq in orexin neurons in the brain (Figure 6A). Pharmacogenetic stimulation with clozapine N-oxide (1 mg/kg) during fasting increased the phosphorylation levels of mTOR and its downstream effectors, p70 S6 kinase and S6 ribosomal protein (Figures 6B–6E), and the mRNA levels of an ER-molecular chaperone, sXbp1 (Figure 6F), in the liver at the 30 min time point. We further investigated whether central administration of orexin A also promotes this adaptive response in the liver. In C57BL/6J mice maintained on NCD, i.c.v. injection of orexin A (3 nmol/mouse) increased the phosphorylation levels of mTOR at 15–30 min and those of p70 S6 kinase (at 15 min) and S6 ribosomal protein (at 15–30 min) in the liver during fasting (Figures 6G–6J). The mRNA levels of sXbp1 were increased 30 min after the i.c.v. injection of orexin A (Figure 6K). Similar orexin A-elicited increases in the phosphorylation levels of mTOR and S6 protein during fasting were observed in HFD-fed WT mice (Figures 7A–7C). These results demonstrated that the central action of orexin A acutely activated the mTOR-sXbp1 pathway in the liver.

Finally, we examined whether the mTOR pathway mediates the anti-inflammatory effect of orexin A under the enhanced ER-stress condition in the liver. WT mice maintained on an HFD or an NCD for 8 weeks were treated daily with either orexin A (0.3 nmol/mouse, i.c.v. at ZT14 for 5 days) or saline and then analyzed under 24 h fasted plus 2 h refed conditions (Figure 7D). The treatment with orexin A prevented a slight increase in CHOP expression and significant elevations of the phosphorylation levels of NF-κB and p38 MAPK in the liver (Figures 7E–7H). Pretreatment with rapamycin, a specific mTOR inhibitor, eliminated these effects of orexin A (Figures 7I–7M), although rapamycin had no effect alone (data not shown). These results indicate that mTOR was involved at least partly in the anti-inflammatory effect of orexin A in the NAFLD liver.

DISCUSSION

The hypothalamus plays central roles in coordination of metabolic functions in peripheral tissues, including the liver; however, the central mechanism for preventing NASH has remained to be identified (Amir et al., 2020). In the present study, we found that...
deficiency of hypothalamic orexin caused severe obesity and the development of NASH with hepatic fibrosis in male and OVX female mice fed an HFD. Prolonged HFD feeding further caused NASH-driven HCC in Ox−/− mice. Supplementation of orexin A prevented NASH-causing abnormalities, including excessive ER stress and chronic inflammation in the liver of Ox−/− mice. Thus, the present study revealed that the lack of orexin causes an accelerated deterioration of NAFLD to fibrosis and HCC and that central orexin, via the autonomic nervous system, can alleviate hepatic ER stress and excessive inflammation.

Physical inactivity due to orexin deficiency is known to cause obesity (Kotz et al., 2017). Narcoleptic patients lacking orexin showed a higher body mass index (Schuld et al., 2000). Consistent with previous studies (Tsuneki et al., 2008, 2016a; Kakizaki et al., 2019), Ox−/− mice showed reduced locomotor activity and excess weight gain under HFD-fed conditions. Since food intake and energy expenditure were comparable between WT and Ox−/− mice, reduction of physical activity appeared to be the main cause of severe obesity in Ox−/− mice when fed an HFD. We further found that short-term (<4-week) exposure to an HFD caused insulin resistance in Ox−/− mice more profoundly than in WT mice. Insulin resistance induces excessive de novo lipogenesis in the liver, leading to development of hepatic steatosis (Buzzetti et al., 2016). Indeed, after 9 weeks of HFD feeding, hepatic triglyceride content was elevated in the liver of Ox−/− mice compared with WT controls. These results suggest that physical inactivity due to orexin deficiency caused obesity and associated insulin resistance, leading to hepatic steatosis.

Simple steatosis can potentially progress to NASH and hepatic fibrosis, and the degree of fibrosis, rather than steatosis, is associated with the increased risk of HCC (Anstee et al., 2019).
Figure 7. Involvement of mTOR in central effect of OXA against hepatic inflammation under HFD-fed condition

(A–C) Male C57BL/6J mice maintained on an HFD for 8 weeks were i.c.v. injected with OXA (3 nmol/mouse) or Sal during fasting, and the isolated livers were subjected to western blot.

(A) Representative western blot images.

(B and C) The levels of p-mTOR (B) and p-S6 (C) 15 min after the i.c.v. injection. n = 6/group. *p < 0.05 determined by Student’s t test.

(D–H) Male WT mice were fed an HFD for 8 weeks, and then OXA (0.3 nmol/mouse, i.c.v.) or Sal was administered for 5 consecutive days at ZT14. The liver was isolated at ZT16 under an enhanced ER-stress condition (i.e., 2 h refeeding after 24 h fasting).

(D) Experimental protocol.

(E) Representative western blot images.

(F) The expression levels of CHOP protein in the liver.

(G and H) The phosphorylation levels of NF-κB (G) and p38 MAPK (H) in the liver. n = 4–5/group. *p < 0.05 determined by one-way ANOVA with Dunnett’s test.

(I–M) Male C57BL/6J mice maintained on an HFD for 8 weeks were pretreated with rapamycin (RAPA; 5 mg/kg, i.p.) for 15 min, and then administered OXA (0.3 nmol/mouse, i.c.v.) or Sal at ZT14 for 5 consecutive days. The liver was isolated at ZT16 under 24 h fasted plus 2 h refed condition.

(I) Experimental protocol.

(J) Representative western blot images.

(K and M) The expression levels of CHOP protein (K), phosphorylated NF-κB (L), and phosphorylated p38 MAPK (M) in the liver. n = 6/group. Data are represented as mean ± SEM.
The present study demonstrated that long-term (>16-week) exposure to an HFD caused the development of NASH and hepatic fibrosis in Ox−/− mice, but not WT, male mice. After prolonged (S2-week) exposure to an HFD, Ox−/− mice showed severe fibrosis and carcinogenesis in the liver. It is unlikely that orexin directly modulates the liver metabolic disorders, since orexin and its receptors are not expressed in the liver (Jöhren et al., 2001). Orexin is produced almost exclusively in the hypothalamic orexin neurons, and circulating levels of orexin are lower than its levels in the cerebrospinal fluid (Heinonen et al., 2008). Therefore, the central actions of orexin are considered to play a major role in the brain-liver network for preventing the progression from simple steatosis to NASH and HCC in obese male mice. Thus, HFD-fed Ox−/− mice are a unique naturally occurring NASH/HCC model based on the brain dysfunction without hyperphagia, unlike previous animal models (Itoh et al., 2011; Imajo et al., 2013).

Increased risks of obesity and NAFLD in postmenopausal women are associated with estrogen deficiency (Lonardo et al., 2019; DiStefano, 2020); however, the role of orexin in the postmenopausal state has remained unknown. The present study demonstrated that OVX alone had no apparent impacts on body weight, tissue weights, and hepatic triglyceride content in female mice after 20 weeks of HFD feeding, whereas orexin deficiency significantly increased them. Increase in the NAS and marked progression of hepatic fibrosis were caused by the combination of OVX and orexin deficiency. These results suggest that estrogen per se had little effect against NASH development but enhanced the protective effects of orexin in female mice after 20 weeks of HFD feeding, whereas orexin deficiency significantly increased them. Increase in the NAS and marked progression of hepatic fibrosis were caused by the combination of OVX and orexin deficiency. These results suggest that estrogen per se had little effect against NASH development but enhanced the protective effects of orexin in female obese mice. Thus, HFD-fed Ox−/− mice are a unique naturally occurring NASH/HCC model based on the brain dysfunction without hyperphagia, unlike previous animal models (Itoh et al., 2011; Imajo et al., 2013).

Under obese conditions, continuous fatty acid overload to the liver causes hepatic ER stress, which induces chronic inflammation, thereby leading to development of NASH (Baiceanu et al., 2016). Hepatic ER stress has also been regarded as a major contributor to the development of NASH-driven HCC (Rebe and Febbraio, 2019), although other factors that can lead to hepatic metabolic reprogramming and chronic inflammation are also involved in NAFLD-associated carcinogenesis (Anstee et al., 2019). In the present study, Ox−/− mice fed an HFD showed increased ER stress in the liver. Daily i.c.v. administration of orexin A suppressed ER stress and associated chronic inflammation in the liver without affecting body weight and hepatic triglyceride content in Ox−/− mice fed an HFD. The beneficial effects of i.c.v.-administered orexin A in the liver were blocked by hexamethonium, an autonomic ganglion blocker. These results suggest that the central actions of orexin attenuated hepatic ER stress through the autonomic nervous system, thereby preventing NASH and HCC under obese conditions.

What is the mechanism underlying the beneficial effects of orexin? The hypothalamic orexin system promotes sympathetic regulation of hepatic metabolic functions (Tsuneki et al., 2015, 2018). Activation of the sympathetic nervous system that innervates the liver has been reported to stimulate hepatic mTOR signaling and Xbp1 splicing in mice when exposed to food-related sensory stimuli during fasting (Brandt et al., 2018). mTOR is a core component of two different kinase complexes, mTORC1 and mTORC2 (Han and Wang, 2018). Because mTORC1 is one of the contributors to hepatic de novo lipogenesis and is over-activated in the liver under obese conditions (Han and Wang, 2018), this protein complex has long been considered as a potential target for treatment of NAFLD (Arora et al., 2022). However, recent studies have demonstrated that mTORC1 has a different role in early and late phases of NAFLD development. Although it promotes benign hepatic steatosis, mTORC1 activation in the progressed NASH liver rather improved steatosis, inflammation, and fibrosis by enhancing very LDL-triacylglycerol secretions (Quinn et al., 2017; Uehara et al., 2022). Prolonged mTORC1 inhibition enhanced hepatic tumorigenesis in lean and obese mice (Umemura et al., 2014). Moreover, the downstream effector sXBP1 acts as a key molecule to adapt for ER stress in most cases (Huang et al., 2019), and mild ER stress that promotes sXBP1 expression serves as a preconditioning stimulus to alleviate subsequent excessive ER stress (Tabas and Ron, 2011). In the present study, we found that i.c.v.-administered orexin A acutely activated the mTOR-sXBP1 pathway in the liver during fasting. Daily administration of orexin A suppressed hepatic inflammation during refeeding after fasting in a rapamycin-dependent manner. Therefore, we suggest that the central action of orexin may prime the hepatic adaptive mechanism against anticipated ER stress through the sympathetic nervous system-hepatic mTOR pathway before eating, thereby preventing excessive ER stress and inflammation in the liver during refeeding.

Alternatively, some extrahepatic mechanism may underlie the relationship between orexin and NASH. In the present study, chronic inflammation in the eWAT and liver occurred almost simultaneously during short-term (8- to-9-week) exposure of Ox−/− mice to an HFD. WAT-derived free fatty acids, adipocytokines, and/or miRNAs are known to be involved in the development of NAFLD (Buzzetti et al., 2016; Korf et al., 2019). Among them, HFD-fed Ox−/− mice showed abnormal hepatic miRNA profiles that were associated with NAFLD, although serum levels of NEFAs and TNF-α were unaltered. Because WAT is the main source of circulating exosomal miRNAs (Thomou et al., 2017), and because the miRNAs secreted from obese adipose tissue macrophages cause insulin resistance in the liver and muscle (Ying et al., 2017), the adipose tissue-derived miRNAs may contribute at least partly to NASH development in Ox−/− mice fed an HFD. However, adipose tissue inflammation in HFD-fed Ox−/− mice was not improved by i.c.v.-administered orexin A, unlike hepatic inflammation. Inflammatory status in the adipose tissue of Ox−/− mice may be more closely related to the degree of obesity rather than autonomic dysregulation. Further investigations might reveal the orexin regulation of inflammatory crosstalk among the liver and other peripheral tissues.
Exercise is recommended to prevent NASH progression under obese conditions (Febbraio et al., 2019). Hypothalamic orexin neurons are activated during aerobic exercise (Shiuchi et al., 2019). The plasma levels of orexin A have been reported to be elevated after 45 min of aerobic exercise in patients with metabolic syndrome (Monda et al., 2020). Similar increase in the plasma orexin A by exercise has been observed in healthy humans (Messina et al., 2016). Because orexin contributes to the induction of motivated behaviors (Baimel et al., 2015), voluntary wheel running activity, an index of aerobic exercise, was reduced in orexin knockout mice (España et al., 2007). The anti-obesity effect of wheel running was reduced in orexin neuron-ablated male mice, compared with WT controls, under an HFD-fed condition (Kakizaki et al., 2019). Thus, exercise intervention for the treatment of obesity seems to be supported by endogenous orexin actions. Given that orexin is secreted with daily rhythm (Tsuneki et al., 2018), we anticipate that timed exercise might be an effective non-pharmacological intervention to enhance the orexin actions to prevent obesity and NAFLD/NASH.

Although the present study focused on the metabolic significance of the orexin regulations of physical activity and autonomic nervous system, orexin is also known to play crucial role in the regulation of sleep and wakefulness. Since orexin stabilizes the wakefulness state, orexin deficiency causes narcolepsy, a sleep disorder associated with excessive daytime sleepiness (Sakurai and Mieda, 2011). Daytime sleepiness is associated with reduced physical activity in older adults and children (Chasens et al., 2007; Isa et al., 2019) and is positively linked to disease severity in patients with NASH (Bemsmeier et al., 2019). Insufficient sleep and sleep disturbances are associated not only with obesity (Reutrakul and Van Cauter, 2018) but also with the development and progression of NAFLD in humans (Marin-Alejandre et al., 2019; Okamura et al., 2019). Exposure of HFD-fed Apoe<sup>−/−</sup> mice to sleep fragmentation has been reported to reduce the orexin A levels, thereby exacerbating monocytes and atherosclerosis (McAlpine et al., 2019), which is a cardiovascular disorder closely linked to NAFLD (Stols-Gonçalves et al., 2019). Therefore, it appears worthwhile to explore the protective effects of orexin against NAFLD/NASH and associated disorders from the viewpoints of sleep-wake regulation.

In conclusion, the present study demonstrated that central actions of orexin play a pivotal role in prevention of NASH and HCC in diet-induced obese mice. Dual actions of orexin, that is, both the anti-obese effect via increase in physical activity and the anti-inflammatory effect in the liver via the autonomic nervous system, appear to underlie the protective mechanisms. Thus, the orexin system may be a crucial target for developing strategy to prevent NASH and HCC.

Limitations of the study

Hypothalamic orexin-producing neurons are distributed throughout the brain, except the cerebellum (Sakurai and Mieda, 2011), and several neural pathways that mediate orexin-induced metabolic regulations have been identified (Tsuneki et al., 2012); however, how the whole-brain orexin system prevents NASH development remains unknown. Also, we cannot exclude the possible involvement of additional unknown hepatic mechanisms other than the autonomic nervous system-hepatic mTOR-XBP1s pathway in the anti-NASH effect of orexin. Moreover, whether long-term orexin A supplementation prevents carcinogenesis in progressed NASH liver remains to be determined due to our current technical difficulties. Finally, since the present study examined the influence of orexin deficiency only in mice, the relationship between orexin-deficient narcolepsy and NASH/HCC in human remains to be clarified.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animals
  - Experimental design
- **METHOD DETAILS**
  - Intracerebroventricular injection
  - Pharmacogenetic activation of orexin neurons
  - Measurements of locomotor activity and energy expenditure
  - Glucose and insulin tolerance tests
  - Analysis of serum parameters
  - Measurement of hepatic triglyceride content
  - Analysis of oxidative stress
  - Western blotting
  - Reverse transcription-quantitative PCR
  - GeneChip analysis of microRNA expression
  - Flow cytometry
  - Histological analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111497.

ACKNOWLEDGMENTS

This study was supported by JSPS KAKENHI grant numbers JP15K09380 (to H.T.), JP15K15599 (to T. Sasaoka), JP19H05011 (to T. Sasaoka), JP21K19704 (to H.T.), and JP22H03506 (to H.T.); JSPS and ISF under the Japan-Israel Research Cooperative Program (to T. Sasaoka); Toyama Pharmaceutical Valley Development Consortium (to T. Sasaoka); JST Moonshot R&D grant number JPMJMS2021 (to T. Sasaoka); JST SPRING grant number JPMJSP2145 (to T.M.); JST-the establishment of university fellowships toward the creation of science technology innovation grant number JPMJFS2115 (to M.S.); and Tamura Science and Technology Foundation (to T. Sasaoka). We thank Y. Numone, T. Asaoka, Y. Matsuoka, T. Sekine, K. Honda, S. Masui, T. Matsushima, Dr. M. Nomoto, and Dr. K. Inokuchi (University of Toyama, Japan) for their technical assistance.

AUTHOR CONTRIBUTIONS

Conceptualization and planning of experiments, H.T. and T. Sasaoka; methodology, A.R., D.K., T. Sakurai, M.Y., A.Y., and T.W.; investigation, T.M., S.T.,...
DiStefano, J.K. (2020). NAFLD and NASH in postmenopausal women: implications for diagnosis and treatment. Endocrinology 161, bqa134.

España, R.A., McCormack, S.L., Mochizuki, T., and Scammell, T.E. (2007). Running promotes wakefulness and increases cataplexy in orexin knockout mice. Sleep 30, 1417–1425.

Febbraio, M.A., Reibe, S., Shalapour, S., Ooi, G.J., Watt, M.J., and Karin, M. (2019). Preclinical models for studying NASH-driven HCC: how useful are they? Cell Metab. 29, 18–26.

Funato, H., Tsal, A.L., Willie, J.T., Kisanuki, Y., Williams, S.C., Sakurai, T., and Yanagisawa, M. (2009). Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity. Cell Metab. 9, 64–76.

Garland, T., Jr., Schultz, H., Chappell, M.A., Keeney, B.K., Meek, T.H., Copes, L.E., Acosta, W., Drenowatz, C., Maciel, R.C., van Dijk, G., et al. (2011). The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. J. Exp. Biol. 214, 206–229.

Gjorgieva, M., Sobolewski, C., Dollick, D., Correia de Sousa, M., and Foti, M. (2019). miRNAs and NAFLD: from pathophysiology to therapy. Gut 68, 2065–2079.

Han, J., and Wang, Y. (2018). mTORC1 signaling in hepatic lipid metabolism. Protein Cell 9, 145–151.

Heenen, M.V., Purhonen, A.K., Mäkelä, K.A., and Herzig, K.H. (2008). Functions of orexins in peripheral tissues. Acta Physiol. 192, 471–485.

Huang, S., Xing, Y., and Liu, Y. (2019). Emerging roles for the ER stress sensor IRE1α in metabolic regulation and disease. J. Biol. Chem. 294, 18726–18741.

Imao, K., Yoneda, M., Kessoku, T., Ogawa, Y., Maeda, S., Sumida, Y., Hyogo, H., Eguchi, Y., Wada, K., and Nakajima, A. (2013). Rodent models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. Int. J. Mol. Sci. 14, 21833–21857.

Ito, T., Sugimoto, T., Murata, S., Tsuboi, Y., Ebina, A., Kondo, Y., Torizawa, K., Okumura, M., Shigemoto, C., Matsuda, N., et al. (2019). Lower physical activity is associated with daytime sleepiness in children aged 9-12 years. J. Child Health Care 23, 415–424.

Ishikawa, A., Wada, T., Nishimura, S., Ito, O., Okekawa, A., Onogi, Y., Watanabe, E., Sameshima, A., Tanaka, T., Tsuneki, H., et al. (2020). Estrogen regulates sex-specific localization of regulatory T cells in adipose tissue of obese female mice. PLoS One 15, e0230885.

Itou, M., Suganami, T., Nakagawa, N., Tanaka, M., Yamamoto, Y., Kamei, Y., Terai, S., Sakaida, I., and Ogawa, Y. (2011). Melanocortin 4 receptor-deficient mice as a novel mouse model of nonalcoholic steatohepatitis. Am. J. Pathol. 179, 2454–2463.

Jöhrn, O. (2018). Orexins/hypocretins and sex. Peptides 99, 115–116.

Jöhrn, O., Neidert, S.J., Kummer, M., Dendorfer, A., and Dominik, P. (2001). Prepro-orexin and orexin receptor mRNAs are differentially expressed in peripheral tissues of male and female rats. Endocrinology 142, 3324–3331.

Kakizaki, M., Tsuneoka, Y., Takase, K., Kim, S.J., Choi, J., Ikkyu, A., Abe, M., Sakimura, K., Yanagisawa, M., and Funato, H. (2019). Differential roles of each orexin receptor signaling in obesity. iScience 20, 1–13.

Kamada, Y., Kiso, S., Yoshida, Y., Chatani, N., Kizu, T., Hamano, M., Tsubakio, M., Takekura, T., Ezaki, H., Hayashi, N., and Takehara, T. (2011). Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet. Am. J. Physiol. Gastrointest. Liver Physiol. 307, G1031–G1043.

Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Toberson, M.S., Unalp-Arida, A., et al.; Nonalcoholic Steatohepatitis Clinical Research Network (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41, 1313–1321.

Korf, H., Boesch, M., Meelberghs, L., and van der Merwe, S. (2019). Macrophages as key players during adipose tissue-liver crosstalk in nonalcoholic fatty liver disease. Semin. Liver Dis. 39, 291–300.

Kotz, C.M., Perez-Leighton, C.E., Teske, J.A., and Billington, C.J. (2017). Spontaneous physical activity defends against obesity. Curr. Opin. 6, 362–370.
Tabas, I., and Ron, D. (2011). Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat. Cell Biol. 13, 184–190.

Thomou, T., Mori, M.A., Dreyfuss, J.M., Konishi, M., Sakaguchi, M., Wolfrum, C., Rao, T.N., Winnay, J.N., Garcia-Martín, R., Grinspoon, S.K., et al. (2017). Adipose-derived circulating miRNAs regulate gene expression in other tissues. Nature 542, 450–455.

Tsukine, H., Murata, S., Anzawa, Y., Soeda, Y., Tokai, E., Wada, T., Kimura, I., Yanagisawa, M., Sakurai, T., and Sasaoka, T. (2008). Age-related insulin resistance in hypothalamus and peripheral tissues of orexin knockout mice. Diabetologia 51, 657–667.

Tsukine, H., Nagata, T., Fujita, M., Kon, K., Wu, N., Takatsuki, M., Yamaguchi, K., Wada, T., Nishijo, H., Yanagisawa, M., et al. (2018a). Nighttime administration of nicotine improves hepatic glucose metabolism via the hypothalamic orexin system in mice. Endocrinology 157, 195–206.

Tsukine, H., Sasaoka, T., and Sakurai, T. (2018b). Sleep control, GPCRs, and glucose metabolism. Trends Endocrinol. Metab. 27, 633–642.

Tsukine, H., Tokai, E., Nakamura, Y., Takahashi, K., Fujita, M., Asaoka, T., Kon, K., Anzawa, Y., Wada, T., Takasaki, I., et al. (2015). Hypothalamic orexin prevents hepatic insulin resistance via daily bidirectional regulation of autonomic nervous system in mice. Diabetes 64, 459–470.

Tsukine, H., Tokai, E., Sugawara, C., Wada, T., Sakurai, T., and Sasaoka, T. (2013). Hypothalamic orexin prevents hepatic insulin resistance induced by social defeat stress in mice. Neuropeptides 47, 213–219.

Tsukine, H., Wada, T., and Sasaoka, T. (2012). Role of orexin in the central regulation of glucose and energy homeostasis. Endocr. J. 59, 365–374.

Tsukine, H., Wada, T., and Sasaoka, T. (2018). Chronopathophysiological implications of orexin in sleep disturbances and lifestyle-related disorders. Pharmacol. Ther. 186, 25–44.

Uehara, K., Sostre-Colón, J., Gavin, M., Santoleri, D., Leonard, K.A., Jacobs, R.L., and Titchenell, P.M. (2022). Activation of liver mTORC1 protects against NASH via dual regulation of VLDL-TAG secretion and de novo lipogenesis. Cell Mol. Gastroenterol. Hepatol. 15, 308–322.

Umemura, A., Park, E.J., Taniguchi, K., Lee, J.H., Shalapour, S., Valasek, M.A., Aghajan, M., Nakagawa, H., Seki, E., Hall, M.N., and Karrin, M. (2014). Liver damage, inflammation, and enhanced tumorigenesis after persistent mTORC1 inhibition. Cell Metab. 20, 133–144.

Wada, T., Hoshino, M., Kimura, Y., Ojima, M., Nakano, T., Tsukine, H., and Sasaoka, T. (2011). Both type I and II IFN induce insulin resistance by inducing different isoforms of SOCS expression in 3T3-L1 adipocytes. Am. J. Physiol. Endocrinol. Metabol. 300, E1112–E1123.

Wada, T., Miyashita, Y., Sasaki, M., Aruga, Y., Nakamura, Y., Ishii, Y., Sasaoka, M., Kanasaki, K., Kitada, M., Koya, D., et al. (2013). Eplerenone ameliorates the phenotypes of metabolic syndrome with NASH in liver-specific Srebp-1c Tg mice fed high-fat and high-fructose diet. Am. J. Physiol. Endocrinol. Metabol. 309, E1415–E1425.

Ying, W., Riopel, M., Bandypadhyay, G., Dong, Y., Birmingham, A., Seo, J.B., Ofrecio, J.M., Wollam, J., Hernandez-Carvajal, A., Fu, W., et al. (2017). Adipose tissue macrophage-derived exosomal miRNAs can modulate in vivo and in vitro insulin sensitivity. Cell 171, 372–384, e12.

Younossi, Z., Anstee, Q.M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., and Bugianesi, E. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. Nat. Rev. Gastroenterol. Hepatol. 15, 11–20.

Younossi, Z.M., Golabi, P., de Avila, L., Paik, J.M., Srishord, M., Fukushima, N., Qiu, Y., Burns, L., Aflendi, A., and Nader, F. (2019). The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: a systematic review and meta-analysis. J. Hepatol. 71, 790–801.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-β-Actin antibody | Cell Signaling Technology | Cat#3700, RRID: AB_2242334 |
| Anti-mTOR antibody   | Cell Signaling Technology | Cat#2972, RRID: AB_330978 |
| Anti-phospho-mTOR (Ser2448) antibody | Cell Signaling Technology | Cat#2971, RRID: AB_330970 |
| Anti-p70 S6 Kinase antibody | Cell Signaling Technology | Cat#9202, RRID: AB_331676 |
| Anti-phospho-p70 S6 Kinase (Thr389) antibody | Cell Signaling Technology | Cat#9205, RRID: AB_330944 |
| Anti-Ribosomal S6 antibody | Cell Signaling Technology | Cat#2212 |
| Anti-phospho-Ribosomal S6 (Ser235/236) antibody | Cell Signaling Technology | Cat#2211, RRID: AB_331679 |
| Anti-elf2α antibody | Cell Signaling Technology | Cat#9721, RRID: AB_2230924 |
| Anti-phospho-elf2α (Ser51) antibody | Cell Signaling Technology | Cat#9721, RRID: AB_330951 |
| Anti-DDIT/CHOP/GADD153 antibody | Santa Cruz Biotechnology | Cat#sc-7351, RRID: AB_627411 |
| Anti-NF-κB p65 antibody | Santa Cruz Biotechnology | Cat#sc-372-G |
| Anti-NF-κB p65 (Ser536) antibody | Santa Cruz Biotechnology | Cat#3033, RRID: AB_331284 |
| Anti-JNK antibody | Santa Cruz Biotechnology | Cat#9251, RRID: AB_331659 |
| Anti-phospho-JNK (Thr183/Tyr185) antibody | Cell Signaling Technology | Cat#9251, RRID: AB_331659 |
| Anti-Mouse CD11c antibody (N418) | AbD Serotec | Cat#MCA1369, RRID: AB_324490 |
| Anti-Mouse IgG-Horseradish Peroxidase | Cytiva | Cat#NA931, RRID: AB_772210 |
| Anti-Rabbit IgG, HRP-conjugated | Cytiva | Cat#NA934, RRID: AB_772206 |
| Anti-Goat IgG-HRP | Santa Cruz Biotechnology | Cat#sc-2020, RRID: AB_631728 |
| CD11c monoclonal antibody (N418), APC | eBioscience | Cat#17-0114-82, RRID: AB_469346 |
| Anti-Goat IgG-HRP | Santa Cruz Biotechnology | Cat#sc-2020, RRID: AB_631728 |
| Anti-Goat IgG-HRP | Santa Cruz Biotechnology | Cat#sc-2020, RRID: AB_631728 |
| Anti-Goat IgG-HRP | Santa Cruz Biotechnology | Cat#sc-2020, RRID: AB_631728 |
| Can Get Signal Solution 1 & 2 | Toyobo | Cat#NKB-101 |
| Chemi Lumi One L | Nacalai Tesque | Cat#07880-70 |
| Prestained SDS-PAGE Standards, broad range | Bio-Rad | Cat#1610318 |
| Prestained Plus Protein Dual Color Standards | Bio-Rad | Cat#1610374 |
| Wide-View Prestained Protein Size Marker II | Fujifilm Wako Pure Chemical | Cat#230-02461 |
| BlueEasy Prestained Protein Marker | Nippon Genetics | Cat#NE-MWP06 |
| D(-)-fructose | Wako | Cat#127-02765 |
| medetomidine | Nippon Zenyaku Kogyo | Cat#NGTD, Domitor |
| midazolam | Sandoz | Cat#MATM |
| butorphanol | Meiji Seika Pharma | Cat#MATB, Vetorphale |
| D(+)-glucose | Wako | Cat#041-00595 |

(Continued on next page)
Continued

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| insulin                  | Eli Lilly Japan | Humulin R |
| TRIsure                  | Nippon Genetics | Cat#BIO-38032 |
| paraformaldehyde         | Fujifilm Wako | Cat#162–16065 |
| Sirius Red               | Polysciences, Inc. | Cat#09400–25 |
| Fast Green FCF           | Fujifilm Wako | Cat#069–00032 |
| 2,4,6-trinitrophenol (picric acid) | Fujifilm Wako | Cat#209–08675 |
| 7-AAD                    | eBioscience | Cat#00-6993-50 |
| orexin A                 | Peptide Institute, Inc. | Cat#4346S |
| clozapine N-oxide        | Cayman Chemical | Cat#16882 |

Critical commercial assays

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| insulin ELISA kit        | Morinaga Institute of Biological Science | Cat#MS302 |
| leptin ELISA kit         | Morinaga Institute of Biological Science | Cat# MS333 |
| NEFA C-Test Wako         | Fujifilm Wako | Cat#279–75401 |
| Transaminase CII-Test Wako | Fujifilm Wako | Cat#431–30901 |
| LBIS mouse TNF-α ELISA kit | Fujifilm Wako | Cat#634–44721 |
| Triglyceride E-Test Wako | Fujifilm Wako | Cat#432–40201 |
| TBARS assay kit          | Cayman Chemical | Cat#700870 |
| PrimeScript RT reagent Kit for Perfect Real Time | Takara Bio | Cat#RR037A |
| TB Green Premix Ex Taq II | Takara Bio | Cat#RR820A |
| miRNeasy Mini Kit        | Qiagen | Cat#217004 |
| FlashTag Biotin HSR RNA Labeling Kit | Thermo Fisher Scientific | Cat#901910 |
| GeneChip miRNA 4.0 Array | Thermo Fisher Scientific, Affymetrix | Cat#902445 |
| Glucose meter: Freestyle Freedom | Abbott Japan | Cat#70959–70 |

Experimental models: Organisms/strains

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| Mouse: C57BL/6J          | Japan SLC | C57BL/6JJmsSlc |
| Mouse: Ox⁻/⁻             | Chemelli et al. (1999) | N/A |
| Mouse: Orexin-flippase knock-in | Chowdhury et al. (2019) | N/A |

Oligonucleotides

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| Primers for reverse transcription-quantitative PCR | This paper | N/A |

Software and algorithms

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| ImageJ                   | Schneider et al. (2012) | https://imagej.nih.gov/ij/ |
| BZ-H4A 1.1.2.4           | Keyence | https://www.keyence.co.jp/products/microscope/fluorescence-microscope/bz-x700/models/bz-h4a/ |
| Transcriptome Analysis Console (TAC) Software, Version 4.0 | Thermo Fisher Scientific | https://www.thermofisher.com/jp/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/affymetrix-transcriptome-analysis-console-software.html |
| Ingenuity Pathway Analysis (IPA) | Qiagen | https://www.qiagen.com/us/products/discovery-and-translational-research/next-generation-sequencing/informatics-and-data/interpretation-content-databases/ingenuity-pathway-analysis/ |
| IBM SPSS Statistics 27   | IBM | https://www.ibm.com/support/pages/spss-statistics-27-now-available |
| GraphPad Prism 4          | GraphPad Software Inc. | https://www.graphpad.com/scientific-software/prism/ |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Toshiyasu Sasaoka (tsasaoka@pha.u-toyama.ac.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). Ox−/− and Ox+/− mice (N11 generation backcrossed to C57BL/6J mice) and heterozygous Orexin-flippase knock-in (Orexin-Flp) mice were prepared as described previously (Chemelli et al., 1999; Tsunek et al., 2015; Chowdhury et al., 2019). Mice were housed under a specific pathogen-free condition at 20–26°C with 12-h light/12-h dark cycle [Zeitgeber time (ZT) 0 = lights on at 7:00, ZT12 = lights off at 19:00], and maintained on a normal chow diet (PicoLab Rodent Diet 20). To cause diet-induced obesity, mice were fed a 60 kcal% high fat diet (HFD; D12492, Research Diets, New Brunswick, NJ) alone or exposed to 60 kcal% HFD with high fructose [30% (w/v)]-containing drinking water (HFFD), according to the protocol described previously (Wada et al., 2013). To investigate the influence of ovariectomy, female mice were ovariectomized using electrocautery or sham-operated under anesthesia with a mixture of medetomidine (0.75 mg/kg, i.p.), midazolam (4 mg/kg, i.p.), and butorphanol (5 mg/kg, i.p.), and recovered for 7 days before experiments. All experimental procedures used in this study were approved by the Committee of Animal Experiments at the University of Toyama (No. A2015PHA-8, A2018PHA-19, A2018PHA-20, A2021PHA-12, A2021PHA-16).

Experimental design
In the experiments shown in Figures 1, 3, 4, S1–S4, and S5, male WT, Ox+/−, and/or Ox−/− mice (6–14 weeks old) were fed NCD or HFD for 2–52 weeks. In the experiments shown in Figure S2, female WT and Ox−/− mice (8–10 weeks old) were fed NCD or HFD for 24 weeks. In the experiments shown in Figures 2, S3, S4C, and S4D, sham-operated WT, OVX-WT, sham-operated Ox−/−, and OVX-Ox−/− female mice (9–11 weeks old) were fed NCD, HFD, or HFFD for 20 weeks. Glucose and insulin tolerance tests were conducted at indicated time during the obeseogenic diet feeding. At the end of the diet feeding, mice were killed by cervical dislocation under 6 h-fasting (Figures 1, 2, 3, 4, and S1–S4) or ad lib fed condition (Figure S5). Then, tissues were immediately isolated.

In the experiments to examine the acute effects of intracerebroventricular injection (ICV) of orexin A on hepatic mTOR signaling (Figures 6G–6K and 7A–7C), C57BL/6J mice (8–9 weeks old) were maintained on NCD or HFD for 8 weeks, and then habituated for 2 days by daily ICV injection of saline; subsequently, the mice were fasted for 16 h, and then ICV-injected with orexin A (3 nmol/mouse, 1 μL/mL) or saline at ZT6. The liver was isolated before and 15 and 30 min after the ICV injection. Moreover, in the experiments to examine the impacts of the DREADD activation of orexin neurons on liver functions (Figures 6A–6F), Orexin-Flp mice expressing hM3Dq in orexin neurons (12 weeks old), maintained on NCD, were fasted for 16 h, and injected with clozapine N-oxide (CNO, 1 mg/kg, i.p.). The liver was isolated before and 30 min after the CNO injection.

In the experiments to examine the chronic effects of ICV-orexin A on hepatic and adipose tissue inflammation under obese condition (Figures 5A–5E, 6A, and 6B), male WT and Ox−/− mice (8–11 weeks old) were fed HFD for 8 weeks, habituated for 2 days by daily ICV injection of saline, and then administered orexin A (0.3 nmol/mouse, 1 μL/mL) or saline for 5 consecutive days at ZT14. The liver and eWAT were isolated at ZT6 under ad lib fed condition.

To enhance hepatic ER stress, mice were reared for 2 h after 24-h fasting, according to the previous study (Lee et al., 2011). To examine the effect of orexin A under the enhanced ER stress condition (Figures 5F–5L and 7D–7M), male WT and Ox−/− mice (8–11 weeks old) were fed HFD or NCD for 8 weeks, and male C57BL/6J mice (8 weeks old) were fed HFD. Thereafter, they were habituated for 2 days by daily ICV injection of saline, and then administered orexin A (0.3 nmol/mouse, 1 μL/mL) or saline or for 5 consecutive days at ZT14 under ad lib fed condition, except last day; namely, after four times of orexin A/saline injection, the mice were fasted for 24 h, and administered the last ICV injection of orexin A or saline at ZT14, and then refeed HFD for 2 h. The liver was isolated at ZT16. To examine whether hexamethonium inhibits the effect of orexin A under the enhanced ER stress condition (Figures 5J–5L), hexamethonium (30 mg/kg) or saline was intraperitoneally injected 15 min before every ICV administration of orexin A or saline. In the experiment to analyze the interaction between rapamycin and orexin A treatment under the enhanced ER stress condition (Figures 7I–7M), rapamycin (5 mg/kg) or its vehicle (75% saline, 10% ethanol, 10% PEG300, and 5% Tween80; Brandt et al., 2018) was intraperitoneally injected 15 min before every ICV administration of orexin A or saline.
METHOD DETAILS

Intracerebroventricular injection
ICV injection was performed as described previously (Tsuneki et al., 2015). In brief, a guide cannula was implanted into the right lateral ventricle (0.5 mm caudal from the bregma, 0.9 mm lateral to the midline, 2.2 mm below the skull surface) of male mice under anesthesia with a mixture of medetomidine (0.75 mg/kg, i.p.), midazolam (4 mg/kg, i.p.), and butorphanol (5 mg/kg, i.p.). Mice were recovered in 7 days, and then subjected to ICV injection of orexin A (1 μL, 0.3 nmol/mouse; 4346S, Peptide Institute, Inc., Osaka, Japan) or saline.

Pharmacogenetic activation of orexin neurons
Male heterozygous Orexin-Flp mice were anesthetized by intraperitoneal injection of a mixture of medetomidine (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), and placed in a stereotactic instrument (SR-6, Narishige, Tokyo, Japan). To express hM3Dq-receptor (a designer receptor exclusively activated by designer drugs, DREADD) in orexin neurons expressing flippase by the AAV(serotype 9)-CMV-dFRT-hM3Dq-mCherry (600 μL, titer: 1 x 10^{12} particles/mL) was bilaterally injected into the lateral hypothalamus (1.4 mm caudal from the bregma, 0.8 mm lateral to the midline, 5.0 mm below the brain surface) of Orexin-Flp mice with a motorized stereotaxic microinjector (IMS-20, Narishige). After 3 weeks of recovery, the mice were subjected to DREADD experiments with administration of clozapine N-oxide (CNO, 16882, Cayman Chemical, MI, USA; 1 mg/kg, i.p.). To confirm the expression of hM3Dq, the brain was isolated after the DREADD experiments, fixed overnight with 4% paraformaldehyde, and immersed in 30% sucrose-containing phosphate buffered saline at 4°C until use. The brain slices (30 μm) were prepared using cryostat (CM3050S, Leica Biosystems, Wetzlar, Germany), and subjected to detection of fluorescence from mCherry co-expressed with hM3Dq under a microscope (BZ-X800, Keyence, Osaka, Japan) using BZ-H4A software (Keyence).

Measurements of locomotor activity and energy expenditure
A small animal metabolism-measuring system (MK-5000QR; Muromachi Kikai, Tokyo, Japan) was used for real-time measurement of locomotor activity, O₂ consumption, CO₂ production, and energy expenditure. Mice were allowed free access to food and water in the metabolic cage on a 12-h light/12 h dark cycle. Amounts of food consumed for 24 h in each mouse were measured as an index of food intake.

Glucose and insulin tolerance tests
In the glucose tolerance test, mice fasted for 6 h were injected with glucose (1 or 2 g/kg, i.p.). In the insulin tolerance test, mice were deprived from food, and then immediately injected with insulin (Humulin R provided by Eli Lilly Japan, Kobe, Japan; 0.5 or 1 unit/kg, i.p.). Blood samples were obtained from the tail vein, and the glucose levels were measured with a glucose meter (Freestyle Freedom, 70959–70, Abbott Japan, Tokyo, Japan).

Analysis of serum parameters
Mice were fasted for 6 h. Serum samples were prepared by centrifugation (220 x g, 15 min, 4°C) of blood samples collected at ZT7-10, and stored at –80°C until use. Serum levels of insulin, leptin, non-esterified fatty acid (NEFA), alanine (AST) and aspartate transaminase (ALT), and tumor necrosis factor-α (TNF-α) were measured using insulin ELISA kit (MS302, Morinaga Institute of Biological Science, Inc., Yokohama, Japan), leptin ELISA kit (MS333, Morinaga Institute of Biological Science, Inc.), NEFA C-Test Wako (279–75401, Fujifilm Wako, Osaka, Japan), Transaminase CII-Test Wako (431–30901, Fujifilm Wako), and LBIS mouse TNF-α ELISA kit (634–44721, Fujifilm Wako), respectively, using a microplate reader (FilterMax F5, Molecular Devices Japan, Tokyo, Japan), according to the manufacturer’s instructions.

Measurement of hepatic triglyceride content
Mice were fasted for 6 h, and the liver was isolated at ZT7-10 and stored –80°C until use. The hepatic triglyceride contents were measured as described previously (Wada et al., 2013). In brief, lipids were extracted from the tissue specimens, according to the Bligh and Dyer method. Triglyceride levels were measured using a colorimetric kit (432–40201, Triglyceride E-Test Wako, Fujifilm Wako) with 4% paraformaldehyde, and immersed in 30% sucrose-containing phosphate buffered saline at 4°C for 1 day. Then, coronal brain slices (30 μm) were prepared using cryostat (CM3050S, Leica Biosystems, Wetzlar, Germany), and subjected to detection of fluorescence from mCherry co-expressed with hM3Dq under a microscope (BZ-X800, Keyence, Osaka, Japan) using BZ-H4A software (Keyence).

Analysis of oxidative stress
Thiobarbituric acid reactive substance (TBARS) assay was conducted to measure the levels of lipid peroxidation, an index of oxidative stress, in serum and liver tissues isolated under ad lib fed condition. The liver tissues were homogenized in RIPA buffer (4.2 g/L NaF, 50 mM Tris, 5 mM EDTA, 10 mM Na₃PO₄, 0.58% aprotinin, 10 μg/mL leupeptin, 2 mM Na₂VO₃, 1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) and centrifuged (1600 x g, 10 min) at 4°C. Supernatant of the liver samples and serum were immediately subjected to colorimetric analysis using a TBARS assay kit (700870, Cayman Chemical, MI, USA) with a microplate reader (SpectraMax i3, Molecular Devices Japan, Tokyo, Japan), according to the manufacturer’s instructions.
Western blotting
The liver tissues dissected from mice were snap-frozen with liquid nitrogen, and stored at –80 °C until use. Protein extraction, SDS-PAGE, and Western blotting were performed, as described previously (Wada et al., 2011). The antibodies and reagents used are listed in Table S3. Chemilumi One L kit (07880–70, Nacalai, Kyoto, Japan) was used for enhanced chemiluminescence detection. The protein expression levels were analyzed using ImageJ software (Schneider et al., 2012).

Reverse transcription-quantitative PCR
Tissues isolated from mice were snap-frozen with liquid nitrogen, and stored at –80 °C until use. Total RNAs were extracted with TRIsure (BIO-38032, Nippon Genetics, Tokyo, Japan), and their concentrations were measured with NanoDrop 2000 (Thermo Fisher Scientific, MA, USA). The RNA samples (250 ng) were subjected to reverse transcription (RT) using PrimeScript RT reagent Kit for Perfect Real Time (RR037A, Takara Bio, Shiga, Japan). Then, quantitative PCR (qPCR) was conducted with TB Green Premix Ex Taq II (RR820A, Takara Bio) and the Mx3000p/3005p qPCR system (Stratagene, Tokyo, Japan), according to the manufacturer’s instructions. Primer pairs used are shown in Table S4. The PCR condition was as follows: initial denaturation at 94 °C for 10 s; 40–50 cycles of 94 °C for 10 s, 62 °C for 20 s, and 72 °C for 15 s. For quantification, the ratio of the target mRNA level to that of an internal control (18S ribosomal RNA or β-actin mRNA) was calculated in each sample.

GeneChip analysis of microRNA expression
Male WT and Ox/−/− mice (6–8 weeks old) fed HFD for 16 weeks were fasted for 6 h, and then the liver tissues were isolated at ZT7-10, snap-frozen with liquid nitrogen, and stored at –80 °C until use. MicroRNAs (miRNAs) were extracted from the tissue specimens using miRNeasy Mini Kit (217004, Qiagen, Venlo, Netherlands), and labeled using FlashTag Biotin HSR RNA Labeling Kit (901910, Thermo Fisher Scientific, MA, USA), according to the manufacturer’s instructions. The expression levels of miRNAs were measured using GeneChip miRNA 4.0 Array (902445, Affymetrix, Thermo Fisher Scientific), and analyzed using Transcriptome Analysis Console (TAC) software (Version 4.0, Thermo Fisher Scientific) with a false discovery rate (FDR) < 0.05. Diseases/functions related to differentially expressed miRNAs were explored using Ingenuity Pathway Analysis (IPA) software (Qiagen).

Flow cytometry
The eWAT was isolated at ZT6 from mice after ICV injections of orexin A or saline for 5 consecutive days under ad lib fed condition. M1/M2 macrophage polarization in the stromal vascular fraction of eWAT was analyzed by flow cytometry using FACS Aria II (BD Bioscience, CA, USA), as described previously (Sameshima et al., 2015). M1 and M2 macrophages were defined as F4/80+/CD45+/CD11c+ and F4/80+/CD45+/CD11c−, respectively.

Histological analysis
Mice were fasted for 6 h, and the liver and eWAT were isolated at ZT7-10. Histological analyses were performed, as described previously (Wada et al., 2013). In brief, tissues were fixed overnight with 4% paraformaldehyde, embedded into paraffin, and subjected to hematoxylin and eosin (H&E) staining, standard Sirius red/Fast green staining, or immunostaining. NAFLD activity score (NAS) and fibrosis stages between two groups or among more than three groups were compared by the non-parametric Mann-Whitney U test or Scheffé test, respectively. Statistical analyses were performed using an IBM SPSS Statistics 27 software (IBM, NY, USA) and a GraphPad Prism 4 software (GraphPad Software Inc., CA, USA). Data are expressed as mean ± standard error of mean (SEM). The p value less than 0.05 was considered statistically significant.

QUANTIFICATION AND STATISTICAL ANALYSIS
In parametric tests, statistical difference between two groups was determined by two-tailed paired Student’s t-test. Differences among four groups [i.e., two genotypes (WT and Ox/−/−) and diet (NCD and HFD), two genotypes (WT and Ox/−/−) and operations ( sham and OVX), and interaction between ICV-orexin A and intraperitoneal hexamethonium treatments] were evaluated by two-way ANOVA, and the main effect of each independent variable was further analyzed by Bonferroni’s test. If the significant interactions between two variables were detected in the two-way ANOVA, all pairs were compared by an ANOVA with Tukey’s test. Otherwise, differences among more than two groups were evaluated by one-way ANOVA with Dunnett’s test (for three-group comparisons) or Tukey’s test (for five-group comparisons). In non-parametric tests, Chi-square test was used to assess independence of the tumor incidence rates between two groups. The NAS and fibrosis stages between two groups or among more than three groups were compared by the non-parametric Mann-Whitney U test or Scheffé test, respectively. Statistical analyses were performed using an IBM SPSS Statistics 27 software (IBM, NY, USA) and a GraphPad Prism 4 software (GraphPad Software Inc., CA, USA). Data are expressed as mean ± standard error of mean (SEM). The p value less than 0.05 was considered statistically significant.