Orexin receptors 1 and 2 in serotonergic neurons differentially regulate peripheral glucose metabolism in obesity

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The wake-active orexin system plays a central role in the dynamic regulation of glucose homeostasis. Here we show orexin receptor type 1 and 2 are predominantly expressed in dorsal raphe nucleus-dorsal and -ventral, respectively. Serotonergic neurons in ventral median raphe nucleus and raphe pallidus selectively express orexin receptor type 1. Inactivation of orexin receptor type 1 in serotonin transporter-expressing cells of mice reduced insulin sensitivity in diet-induced obesity, mainly by decreasing glucose utilization in brown adipose tissue and skeletal muscle. Selective inactivation of orexin receptor type 2 improved glucose tolerance and insulin sensitivity in obese mice, mainly through a decrease in hepatic gluconeogenesis. Optogenetic activation of orexin neurons in lateral hypothalamus or orexinergic fibers innervating raphe pallidus impaired or improved glucose tolerance, respectively. Collectively, the present study assigns orexin signaling in serotonergic neurons critical, yet differential orexin receptor type 1- and 2-dependent functions in the regulation of systemic glucose homeostasis.
T he obesity epidemic represents a major global socio-economic burden, currently affecting over a third of the world’s population. However, the underlying cellular causes of weight gain and its associated metabolic comorbidities, such as type 2 diabetes mellitus, are still only poorly understood.

Orexins (hypocretins) are neuropeptides expressed by distinct neurons in the lateral hypothalamic area (LHA) and adjacent regions, including the perifornical area, and dorsomedial and posterior hypothalamus in humans and rodents. The orexin system consists of the two distinct neuropeptides orexin-A and orexin-B (hypocretin-1 and -2), which are both derived from a common precursor peptide, and they act on two G-protein coupled receptors, orexin receptor type 1 (Ox1R) and type 2 (Ox2R). Human cerebrospinal fluid (CSF) orexin negatively correlates with serum glucose levels, and narcolepsy patients exhibit low orexin levels in CSF and increased obesity. Ablation correlates with serum glucose levels, and narcolepsy patients activate and recruit thermogenic brown and beige fat and regulate ablation studies have demonstrated that central serotonergic cells mRNA is increased in the arcuate nucleus of the hypothalamus. mRNA in the ventral medial hypothalamic nucleus, while Ox2R

Differential regulatory roles of Ox1R and Ox2R in energy homeostasis and glucose metabolism throughout the organism have been revealed employing pharmacological or genetic methods. One study shows that Ox2R but not Ox1R predominantly mediates the orexin-overexpression-induced amelioration of obesity in mice fed a high-fat diet (HFD). In another study, Ox1R deficiency increases the body weight of mice fed a Chow diet, but decreases intake of HFD and prevents HFD-induced excess of body weight gain, while Ox2R deficient mice mainly exhibited decreased energy expenditure. Moreover, systemic histological studies have shown overlapping but distinct expressions of Ox1R and Ox2R in many brain regions, such as the cerebral neocortex, basal ganglia, hippocampus, thalamus, mid-brain, and reticular formation. Fasting increases Ox1R mRNA in the ventral medial hypothalamic nucleus, while Ox2R mRNA is increased in the arcuate nucleus of the hypothalamus.

Interestingly, within a defined cell population of serotonergic neurons in raphe nuclei (RN), RNA sequencing (RNA-seq) experiments revealed that mainly Ox2R but not Ox1R is differentially expressed among the subpopulations in dorsal raphe nucleus (DR), median raphe nucleus (MR) and caudal raphe nuclei including the raphe pallidus nucleus (RPa). Serotonergic neurons in subregions of RN have been described as very heterogeneous and enriched in dopaminergic, cholinergic, and glutamatergic neuronal subtypes. Importantly, the serotonergic system plays a central role in regulating wakefulness, appetite, glucose metabolism, and energy homeostasis. Pharmacological experiments show that serotonin inhibits food intake and clinical drugs targeting the serotonergic system result in body weight loss. In addition, ablation studies have demonstrated that central serotonergic cells activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis, and injection of orexin-A into RPa produces a sustained increase in brown adipose tissue (BAT) thermogenesis.

In this work, we systematically investigated the expression and distribution of orexin receptors in serotonergic neurons in RN, and the impact of optogenetic activation of orexin signaling and specific inactivation of Ox1R or Ox2R signaling in serotonin transporter (Sert)-expressing cells on glucose metabolism and energy homeostasis. The present study assigns orexin signaling in serotonergic neurons critical, yet differential orexin receptor type 1- and 2-dependent functions in the regulation of systemic glucose homeostasis.

Results

Distribution of Ox1R and Ox2R expression in serotonergic neurons in RN. To systemically define the expression pattern of Ox1R and Ox2R in serotonergic neurons, Slc6a4-cre (Sert-Cre) mice were mated with tdTomato Flox (tdTomato fl/fl) mice to obtain SerttdTomato mice that express the red tomato (tdTomato) reporter protein upon Sert-Cre-mediated recombination of a loxP-flanked stop cassette specifically in serotonergic neurons. Beyond the raphe nuclei, ectopic expression of tdTomato was also detected in deep layers of the cingulate cortex (Cg1 and Cg2), and ventral posterolateral (VPL), and ventral posteromedial (VPM) thalamic nuclei (Supplementary Fig. 1a–h). There were no detectable orexin receptors in tdTomato positive cells in these areas except for moderate expression levels of Ox2R in the cingulate cortex, as revealed by fluorescent in situ hybridization (RNAscope) employing probes directed to tdTomato, Ox1R (Hcrt1), and Ox2R (Hcrt2) mRNA (Supplementary Fig. 1i, k). Most of the Ox2R signal could not be detected in tdTomato positive cells in the cingulate cortex, where only five cells out of the 45 tdTomato positive cells (10.74%) expressed Ox2R (Supplementary Fig. 1j). As a positive control, abundant Ox1R and Ox2R mRNA was expressed in tdTomato positive neurons in DR (Supplementary Fig. 1l). We further confirmed the specificity of Cre expression in raphe nuclei of a SerttdTomato mouse. As markers for serotonergic neurons, tryptophan hydroxylase isoform 2 (TPH2) and Pet1 were co-expressed by 94.90% or all of analyzed cells in DR and RPs, respectively (Supplementary Fig. 1m, n, q). Here, almost all tdTomato positive neurons are TPH2 and/or Pet1 positive in DR, MR and RPs (Supplementary Fig. 1m, p, q). More than 95% of TPH2 and/or Pet1 positive neurons are tdTomato positive (Supplementary Fig. 1m, o, r). This indicates specific and efficient expression of Cre in serotonergic neurons in raphe nuclei.

The endogenous expression of Ox1R and Ox2R in serotonin neurons in RN was systemically investigated in SerttdTomato mice. Ox1R was expressed in 66.01–89.20% of serotonergic neurons (tdTomato positive) in DR, MR, and RPs and the relative fluorescence signal in each neuron was strongest in DR-dorsal (DRD, =1.86) compared to other regions (≈0.64–0.92; Fig. 1a–d). In contrast, Ox2R was expressed in 63.43% of serotonergic neurons in DR-ventral (DRV), which was more than in DRD (≈45.36%), MR-dorsal (MRD, =28.18%), MR-ventral (MRV, =13.94%) and RPs (≈22.94%, Fig. 1a, b, e). The relative Ox2R fluorescence signal in each cell is also stronger in DRV (≈2.66) compared to DRD (≈1.33) and MRD (≈0.60, Fig. 1a, b, f). Of note, the Ox2R signal in serotonin neurons in MRV (≈0.17) and RPs (≈0.24) was very weak (Fig. 1a, b, f).

Next, we analyzed published single-cell RNA sequencing (scRNA-seq) results of serotonergic neurons in DR. 20.74% serotonergic neurons expressed Ox1R but not Ox2R (Ox1R-only), 6.25% serotonergic neurons expressed Ox2R but not Ox1R (Ox2R-only), only 2.27% serotonergic neurons expressed both orexin receptors (Ox1R/Ox2R+), and 70.74% serotonergic neurons expressed none of the orexin receptors (Ox1R/Ox2R−) (Fig. 1g). Interestingly, Skl7a8 (vesicular glutamate transporter 3, VGLUT3) was the only gene showing significantly different expression levels, comparing Ox1R-only with Ox2R-only serotonergic neurons (Source Data of Fig. 1i). The average expression level of VGLUT3 was higher in Ox2R-only than Ox1R-only.
Fig. 1 Distribution of Ox1R and Ox2R in serotonergic neurons of the raphe nucleus of a SERTtdTomato mouse. a Representative images of RNAscope in situ hybridization in the dorsal raphe nucleus (dorsal (DRD), ventral (DRV)), and median raphe nucleus (dorsal (MRD), ventral (MRV)). b Representative images of RNAscope in situ hybridization in the raphe pallidus (RPa). c, d Percentages of Ox1R or Ox2R positive neurons in serotonergic neurons. d, f The average of relative fluorescence intensity of Ox1R or Ox2R signal in each serotonergic neuron. Fluorescence was normalized by the mean of all regions for each mouse. e Positive neurons (%): Ox1R-only (n=146), Ox2R-only (n=44), Ox1/2R (n=16), and Ox1R/Ox2R (n=46). g Scatter plot of raw cell read counts of Ox1R and Ox2R. h Scatter plot of raw vesicular glutamate transporter 3 (VGLUT3) counts against raw Ox1R or Ox2R counts. k, l Heat map of gene counts for cells with column-wise z-scores. Mean gene abundance was calculated and analyzed: k percentages of Ox1R-only, Ox2R-only, Ox1/2R+ and Ox1/2R− serotonergic neurons; percentages of VGLUT3 positive neurons in (l) Ox1R-only and (m) Ox2R-only serotonergic neurons. Gray, tryptophan hydroxylase isoform 2 (TPH2); red, Ox1R; cyan, Ox2R; blue, VGLUT3. n = 3. Source data are provided as a Source Data file.
serotonergic neurons, mainly due to the fact that more Ox2R-only serotonergic neurons are VGLUT3 positive (Fig. 1h, i, l). Source Data of Fig. 1f). Further, we compared the gene expression patterns among Ox1R-only, Ox2R-only, Ox1R/Ox2R+ (both positive), and Ox1R/Ox2R− (both negative) serotonergic neurons, and revealed 47 differently expressed genes (Fig. 1f, Source Data of Fig. 1f).

In scRNAseq analysis, the percentage of orexin-receptor-positive serotonergic neurons was lower than what we detected by RNAscope. Due to different sensitivity of detecting genes between scRNA-seq and RNAscope, Ox1R-only and Ox2R-only serotonergic neurons revealed by scRNA-seq could be similar to Ox1R- and Ox2R-dominant serotonergic neurons revealed by RNAscope. We further confirmed scRNAseq findings using RNAscope combined with TPH2 immunostaining, which revealed 26.11%, 13.89%, 38.89%, and 21.11% Ox1R-only, Ox2R-only, Ox1R/Ox2R+ and Ox1R/Ox2R− serotonergic cells, respectively (Fig. 1j, k). VGLUT3 was mainly expressed in DRV, and more Ox2R-only serotonergic neurons are VGLUT3 positive (88.00%) compared to Ox1R-only serotonergic neurons (12.77%) (Fig. 1j, l, m).

**Selective inactivation of Ox1R and Ox2R in serotonergic neurons in RN.** To specifically inactivate Ox1R or Ox2R in serotonergic neurons (Ox1RASERT, Ox2RASERT), Ox1R Flox (Ox1R fl/fl), and Ox2R Flox (Ox2R fl/fl) mice were generated via homologous recombinant targeting in embryonic stem (ES) cells (Supplementary Fig. 2). The resulting animals were crossed with Sert-Cre mice to obtain Ox1R fl/fl, Sert-Cre tg/wt mice (Ox1RASERT), and Ox2R fl/fl, Sert-Cre tg/wt mice (Ox2RASERT). Mice from the same breeding without the Cre transgene were used as controls (Ox1R fl/fl, Sert-Cre wt/wt or Ox2R fl/fl, Sert-Cre wt/wt mice). Ox1RASERT or Ox2RASERT mice were further intercrossed with Ox1R fl/fl, tdTomato fl/fl mice or Ox2R fl/fl, tdTomato fl/fl mice, respectively, to obtain Ox1R fl/fl, tdTomato fl/fl, Sert-Cre tg/wt (Ox1RASERTtdTomato) mice or Ox2R fl/fl, tdTomato fl/fl, Sert-Cre tg/wt (Ox2RASERTtdTomato) mice that express tdTomato but lack Ox1R or Ox2R in serotonergic neurons. In Ox1RASERTtdTomato or Ox2RASERTtdTomato mice, we confirmed the specific deletion of Ox1R or Ox2R, respectively, in serotonergic neurons in RN via RNAscope detection of tdTomato, Ox1R, and Ox2R mRNA. In Ox1RASERTtdTomato mice, the Ox1R signal was almost completely abrogated in serotonergic neurons and only single-labeled, non-Sert Ox1R-expressing cells were observed, while the Ox2R signal exhibited a similar expression pattern to that in the SerttdTomato mice (Fig. 2a, c). In contrast, in Ox2RASERTtdTomato mice, the Ox1R signal was similar to that in the SerttdTomato mice, while the Ox2R signal almost disappeared in serotonergic neurons and some single-labeled, non-Sert Ox2R-expressing cells were observed (Fig. 2b, d). Accordingly, Ox1RASERTtdTomato mice showed significantly lower percentages of Ox1R positive neurons in serotonergic neurons and also significantly lower raw fluorescence intensity of Ox1R signal in individual serotonergic neurons in all RN regions compared to Ox2RASERTtdTomato mice (Fig. 2e, f). Ox2RASERTtdTomato mice showed significantly lower percentages of Ox2R positive neurons in serotonergic neurons in DRD, DRV, and MRD and also significantly lower raw fluorescence intensity of Ox2R signal in individual serotonergic neurons in DRD and DRV than Ox1RASERTtdTomato mice (Fig. 2g, h). In Ox2RASERTtdTomato mice, Ox1R fluorescence intensity in each DRD serotonergic neuron was highest among all RN regions and significantly higher than that in DRV, MRD, and MRV (Fig. 2f). In Ox1RASERTtdTomato mice, Ox2R mRNA expression levels in serotonergic neurons in DRV were highest and significantly higher than levels in MRD, MRV, and RPa, as quantified in percentages of positive neurons and raw fluorescence intensity (Fig. 2g, h).

Notably, in serotonergic neurons in MRV and RPa, Ox2R mRNA expression levels were similar between Ox1RASERTtdTomato mice and Ox2RASERTtdTomato mice, which indicates that this signal was close to the background fluorescence related to method limitations, presumably (Fig. 2g, h). Therefore, only Ox1R but not Ox2R was detectable in serotonergic neurons in MRV and RPa.

**Differential activation of DR serotonergic neurons by orexin.** To further validate the deletion of orexin receptor signaling, we performed perforated patch-clamp recordings and Ca2+ imaging on DR serotonergic neurons from control, Ox1RASERT, and Ox2RASERT mice with tdTomato reporter or genetically encoded calcium indicator GCaMP6 in serotonergic neurons (Fig. 3a–e). During the experiments, GABAergic and glutamatergic synaptic input was blocked.

In the first series of experiments we performed patch-clamp recordings in the DR with the focus mainly on the DRD. 100 nM orexin A increased the action potential firing rate in control serotonergic neurons (p < 0.0001, n = 21, Fig. 3b), which is in line with previous studies32–34. Similarly, 100 nM orexin A increased the activity of serotonergic neurons in Ox1RASERTtdTomato (p < 0.05, n = 9; Fig. 3b) and Ox2RASERTtdTomato mice (p < 0.0005, n = 11; Fig. 3b). In contrast, 100 nM orexin B increased action potential firing rate only in control (p < 0.0001, n = 27; Fig. 3c) and Ox1RASERTtdTomato mice (p < 0.0001, n = 14; Fig. 3c) but not in Ox2RASERTtdTomato mice (p = 0.28, n = 11; Fig. 3c). A representative recording with response to 1, 10, and 100 nM of orexin A in a control neuron was shown in Supplementary Fig. 3a, b.

In the second series of experiments we focused on the analysis of serotonergic neurons in the DRV using Ca2+ imaging with the genetically encoded calcium indicator GCaMP6 (Fig. 3d, e). 100 nM orexin A increased [Ca2+]i in control (p < 0.0001, n = 159), Ox1RASERT (p < 0.0001, n = 200) and Ox2RASERT neurons (p < 0.0001, n = 75). The increase in [Ca2+]i was significantly smaller in Ox2RASERT neurons compared to control (p < 0.0001) and Ox1RASERT (p < 0.0001) neurons. 100 nM orexin B increased [Ca2+]i in control (p < 0.0001, n = 75) and Ox1RASERT neurons (p < 0.0001, n = 92) and had no effect on Ox2RASERT neurons (p = 0.8, n = 122).

The differential and complex response patterns are likely caused by the differences in affinity profiles of orexin A and orexin B to Ox1R and Ox2R35, and the varying rate of Ox1R and Ox2R expression in different regions of the DR as described above. In radioligand binding assays, orexin-A had high affinities to Ox1R (IC50 = 20 nM) as well as to Ox2R (IC50 = 38 nM), whereas orexin B has a high affinity to Ox2R (IC50 = 36 nM) and only a low affinity to Ox1R (IC50 = 420 nM)4. While both orexin receptors are expressed in the DRD and the DRV, the relative expression of Ox1R is higher in the DRD, whereas the relative expression of Ox2R is higher in the DRV (Fig. 1a, d–f).

Since orexin-A is highly affine to both receptors, orexin A increases activity in serotonergic DRD neurons of Ox1RASERTtdTomato and Ox2RASERTtdTomato mice, even when one receptor is switched off. The effect of Ox1R deletion becomes only detectable at very low orexin A concentrations (Supplementary Fig. 3c) since the affinity of orexin A is higher to Ox1R than to Ox2R. Orexin B, in contrast, has only a low affinity for Ox1R. Thus, when Ox2R are switched off, the neurons do not react anymore (Fig. 3c). In the DRV, the situation is different in the sense that Ox2R are relatively higher expressed than Ox1R. If Ox2R are missing, there are too few receptors to fully activate the serotonergic neurons of the DRV with orexin A. In the absence of Ox2R, orexin B can practically no longer trigger an effect due to its low affinity to Ox1R.
Orexin neurons are activated in HFD-induced obesity. In order to investigate the activation of orexin neurons in lean and obese mice, we compared c-Fos expression in orexin neurons in 6 h-fasted BL/6 mice, which had been fed a HFD for 10 weeks since weaning, to that in mice exposed for the same period to a control diet (CD). To this end, we determined the expression of orexin (Hcrt) and c-Fos (Fos) mRNA via RNAscope hybridization. This analysis revealed, that the proportion of c-Fos-expressing orexin neurons in the LH was

Fig. 2 Specific inactivation of Ox1R or Ox2R in serotonergic neurons of a Ox1RΔSERT/tdTomato or Ox2RΔSERT/tdTomato mouse, respectively. a Representative images of RNAscope in situ hybridization in the dorsal raphe nucleus (dorsal (DRD), ventral (DRV)) and median raphe nucleus (dorsal (MRD), ventral (MRV)) of a Ox1RΔSERT/tdTomato mouse and b a Ox2RΔSERT/tdTomato mouse. c Representative images of RNAscope in situ hybridization in the raphe pallidus (RPa) of a Ox1RΔSERT/tdTomato mouse and d a Ox2RΔSERT/tdTomato mouse. e, g Percentages of Ox1R or Ox2R positive neurons in serotonergic neurons. f, h The average of raw fluorescence intensity of Ox1R or Ox2R signal in each serotonergic neuron. Blue, tdTomato; red, Ox1R; cyan, Ox2R. Scale bar: 100 µm in (a, b) and 50 µm in (c, d). Ox1RΔSERT/tdTomato, n = 4; Ox2RΔSERT/tdTomato, n = 3. Data are represented as means ± SEM. f Ox1RΔSERT/tdTomato, p = 0.0005 (DRV) and 0.0011 (MRD); Ox2RΔSERT/tdTomato, p = 0.013 (MRD), 0.0006 (MRV) and 0.0014 (RPa); Ox2RΔSERT/tdTomato, p = 0.0002 (DRD) and 0.0046 (MRD). h Ox1RΔSERT/tdTomato, p = 0.0038 (MRD), 0.039 (MRV) and 0.037 (RPa); Ox2RΔSERT/tdTomato, p = 0.037 (DRD), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; as determined by two-way ANOVA followed by Sidak’s post hoc test. The main effect of genotype: e (F (1, 25) = 583.60, p < 0.0001), f (F (1, 25) = 234.20, p < 0.0001), g (F (1, 25) = 53.99, p < 0.0001), h (F (1, 25) = 20.08, p = 0.0001). #p < 0.05, ##p < 0.01, ###p < 0.001, compared to DRD (f) or DRV (g, h), as determined by paired two-tailed t-test. Source data are provided as a Source Data file.
significantly increased in mice exposed to HFD compared to CD feeding (Fig. 4a, b). Collectively, these data indicate an increased activation of orexin neurons in obese mice. Then, we investigated the impact of Ox1R or Ox2R inactivation in serotonergic system on energy homeostasis under both normal chow diet (NCD)-fed lean condition and HFD-induced obese condition. There were no differences in body weight (BW; Fig. 4c, g), glucose tolerance under 16-h fasting condition (Fig. 4d, h) or insulin sensitivity under randomly fed condition (Fig. 4f, j) between control and Ox1RΔSERT mice. Two-way ANOVA revealed a significant decrease of glucose tolerance in obese (F(1, 105) = 7.86, p = 0.0060) but not lean Ox1RΔSERT mice, compared to control mice after a 6-h fasting period, though there was no significant change at individual time points by post hoc Tukey tests. Ox1RΔSERT, p = 0.03, n = 9; Ox2RΔSERT, p = 0.0018, n = 11. e Orexin B application: Ctrl, p < 0.0001, n = 27; Ox1RΔSERT, p < 0.0001, n = 14; Ox2RΔSERT, p = 0.28, n = 11. Abbreviation: baseline (BL). d, e Orexin A and orexin B effect on [Ca2+]i of serotonergic neurons in DRV measured with GCaMP6. d Original recordings (top) and heat maps of five individual neurons for each set of experiments (bottom). The recordings show the responses to the orexins and high K+ saline. Dashed lines indicate the range where the responses were quantified. e Calcium responses upon 100 nM orexin A and orexin B. Data are shown as the percentage of the maximal response to high K+ saline. Mean increases in [Ca2+]i between experimental groups were compared, performing ANOVA with post hoc Tukey tests. Orexin A application: Ctrl vs Ox1RΔSERT, p = 0.88; Ctrl vs Ox2RΔSERT, p = 0.0001; Ox1RΔSERT vs Ox2RΔSERT, p < 0.0001 (Ctrl, n = 159; Ox1RΔSERT, n = 200; Ox2RΔSERT, n = 75). Orexin B application: Ctrl vs Ox1RΔSERT, p = 0.30; Ctrl vs Ox2RΔSERT, p < 0.0001; Ox1RΔSERT vs Ox2RΔSERT, p < 0.0001 (Ctrl, n = 75; Ox1RΔSERT, n = 92; Ox2RΔSERT, n = 122). In the box plots, the horizontal lines show the median of the data. Boxes indicate the 25th and 75th percentile. The whiskers were calculated according to the ‘Tukey’ method. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. n values are given in brackets. Source data are provided as a Source Data file.
hoc test (Fig. 4e, i). Similarly, in lean Ox2RΔSERT mice, BW, glucose tolerance, and insulin sensitivity remained unchanged (Fig. 4k–n). Interestingly, in obese Ox2RΔSERT mice, glucose tolerance was significantly improved after a 16-h fasting period (Fig. 4p), while BW and insulin sensitivity remained unchanged, compared to control mice (Fig. 4o, r). Two-way ANOVA revealed a significant improvement of glucose tolerance in obese Ox2RΔSERT mice, compared to control mice after a 6-h fasting period, though there was no significant change at individual time points by post hoc test ($F(1, 100) = 6.08, p = 0.015$, Fig. 4q).

Indirect calorimetry revealed unaltered locomotor activity and daily energy intake in Ox1RΔSERT and Ox2RΔSERT mice, compared to the respective control mice, under both lean and obese conditions (Supplementary Fig. 4). Collectively, these
Fig. 4 Increased c-Fos activation in orexin neurons upon high-fat diet (HFD) feeding and improved glucose tolerance in Ox2R<sup>ΔSERT</sup> mice fed a HFD. a Representative images of RNAscope in situ hybridization in the lateral hypothalamus (LH) of a BL/6 mouse fed a control diet (CD) or HFD. n = 5 (CD) and 4 (HFD). b Percentages of c-Fos positive neurons in orexin neurons. n = 5 (CD) and 4 (HFD). p = 0.029. c, g Average body weight, glucose tolerance test under d, h 16-h and e, i 16-h fasting conditions, and f, j insulin tolerance test of control (Ctrl) and Ox1R<sup>ΔSERT</sup> mice on normal chow diet (NCD) or HFD. NCD-Ctrl and NCD-Ox1R<sup>ΔSERT</sup>, n = 9; HFD-Ctrl, n = 8 (g, h, j) or 11 (i); HFD-Ox1R<sup>ΔSERT</sup>, n = 10 (g, h, j) or 12 (i). i, p = 0.16 at 30 min. k, o Average body weight, glucose tolerance test under l, p 16-h and m, q 6-h fasting conditions, and n, r insulin tolerance test of Ctrl and Ox2R<sup>ΔSERT</sup> mice on NCD or HFD. NCD-Ctrl, n = 11; NCD-Ox2R<sup>ΔSERT</sup>, n = 8; HFD-Ctrl, n = 11 (o, p, r) or 13 (q); HFD-Ox2R<sup>ΔSERT</sup>, n = 9. p = 0.0002 (30 min) and 0.045 (60 min); q = 0.076 (30 min). Magenta, orexin; yellow, c-Fos. Scale bar: 100 µm. Data are represented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; as determined by unpaired two-tailed Student’s t-test (b) or two-way ANOVA followed by Sidak’s post hoc test (i, p, q). Two-way ANOVA revealed a significant main effect of genotype in (i) (F (1, 105) = 7.86, p = 0.0060), p (F (1, 90) = 18.22, p < 0.0001) and q (F (1, 100) = 6.08, p = 0.015). Source data are provided as a Source Data file.

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Fig. 5 Insulin sensitivity is impaired in Ox1R<sup>ΔSERT</sup> mice but improved in Ox2R<sup>ΔSERT</sup> mice fed a HFD in hyperinsulinemic-euglycemic clamp analysis. a Clamp glucose infusion rates (GIR), b hepatic glucose production (HGP) in the basal state and during the steady state of clamp analysis, and c tissue-specific insulin-stimulated [1-14C]-Deoxy-D-glucose uptake in white adipose tissue (WAT), brown adipose tissue (BAT), and skeletal muscle (SM) under steady-state conditions of control (Ctrl) and Ox1R<sup>ΔSERT</sup> mice. HFD-Ctrl, n = 8; HFD-Ox1R<sup>ΔSERT</sup>, n = 14. a, p = 0.0025 (110 min) and 0.0028 (120 min); c p = 0.011 (BAT) and 0.0070 (SM). d Clamp GIR, e HGP in the basal state and during the steady state of clamp analysis, and f tissue-specific insulin-stimulated [1-14C]-Deoxy-D-glucose uptake in WAT, BAT, and SM under steady-state conditions of control (Ctrl) and Ox2R<sup>ΔSERT</sup> mice. HFD-Ctrl, n = 10; HFD-Ox2R<sup>ΔSERT</sup>, n = 11. d, p = 0.034 (90 min), 0.047 (100 min), 0.041 (110 min) and 0.042 (120 min); e p = 0.048. Data are represented as means ± SEM. *p < 0.05, **p < 0.01; as determined by two-way ANOVA followed by Sidak’s post hoc test (d, e) or unpaired two-tailed Student’s t-test (c, f). Two-way ANOVA revealed a significant main effect of genotype in (a) (F (1, 220) = 18.30, p < 0.0001) and (d) (F (1, 209) = 35.39, p < 0.0001). Source data are provided as a Source Data file.

![Figure 5](image-url)

Results indicate that glucose metabolism is improved in obese mice upon selective inactivation of Ox2R in serotonergic neurons.

Insulin-stimulated HGP suppression and tissue glucose uptake in obese Ox1R<sup>ΔSERT</sup> and Ox2R<sup>ΔSERT</sup> mice. To further specifically address the regulation of glucose metabolism in HFD-induced obese Ox1R<sup>ΔSERT</sup> and Ox2R<sup>ΔSERT</sup> mice, we performed hyperinsulinemic-euglycemic clamp studies. The glucose infusion rate (GIR) required to maintain euglycemia during steady-state conditions was significantly reduced in Ox1R<sup>ΔSERT</sup> mice, compared to their littermate control mice (Fig. 5a, Supplementary Fig. 5a). Hepatic glucose production (HGP) was not significantly different between control and Ox1R<sup>ΔSERT</sup> mice under basal clamp conditions or in response to insulin (Fig. 5b). Interestingly, the rate of plasma glucose disappearance (Rd) was significantly decreased in Ox1R<sup>ΔSERT</sup> mice at a steady state while the levels under basal clamp conditions were not significantly different (Supplementary Fig. 5b). Determination of tissue-specific glucose uptake revealed impaired glucose uptake into BAT and skeletal muscle (SM) of obese Ox1R<sup>ΔSERT</sup> mice, while glucose uptake into white adipose tissue (WAT) remained unchanged (Fig. 5c). In contrast, GIR was significantly increased during steady-state conditions in Ox2R<sup>ΔSERT</sup> mice compared to their littermate control mice (Fig. 5d, Supplementary Fig. 5d). Ox2R<sup>ΔSERT</sup> mice exhibited similar HGP levels under basal clamp conditions but significantly lower HGP at steady state, compared to control mice, indicating that the suppression of HGP was more efficient in response to insulin in obese Ox2R<sup>ΔSERT</sup> mice (Fig. 5e). There was no significant difference of Rd or tissue-specific glucose uptake between control and Ox2R<sup>ΔSERT</sup> mice (Fig. 5f, Supplementary Fig. 5e). The insulin infusion was confirmed by detecting human insulin levels in serum at baseline and at the end of hyperinsulinemic-euglycemic clamp studies (Supplementary Fig 5c, f).

![Figure 6](image-url)

Altered liver and BAT in obese Ox1R<sup>ΔSERT</sup> and Ox2R<sup>ΔSERT</sup> mice in hyperinsulinemic-euglycemic clamp studies. To investigate the mechanisms underlying the effect of serotonergic-
specific inactivation of Ox1R or Ox2R in hyperinsulinemic-euglycemic clamp studies, we further analyzed gene and protein expression in the liver and BAT dissected from these animals under clamp steady-state conditions.

In the liver, p-AktSer473 protein, the major downstream effector of the insulin signaling pathway, was significantly increased in Ox2RΔSERT mice but unchanged in Ox1RΔSERT mice, compared to control mice, while the total Akt protein levels remained unchanged (Fig. 6a–d). Glucose-6-phosphatase (G6Pase) protein, which is essential for gluconeogenesis, was significantly decreased in Ox2RΔSERT mice but unchanged in Ox1RΔSERT mice, compared to control mice (Fig. 6a–d).

To investigate the mechanisms of glucose uptake reduction in BAT of obese Ox1RΔSERT mice compared to control mice, we compared the gene expression profiles of BAT dissected under clamp steady-state conditions from Ox1RΔSERT and control mice by total mRNA sequencing. This analysis revealed 265 genes significantly regulated in Ox1RΔSERT mice compared to control mice. The fatty acid elongase genes Elov3 and Elov4, the lipoprotein lipase gene Lpl, the very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 2 gene Hacd2, the mitochondrial genes mt-Nd5 and Ndufa4 involved in Complex I and oxidative phosphorylation, the uncoupling protein 1 gene Ucp1 and the insulin receptor substrate 1 gene Irs1 were decreased in expression (Fig. 6e).

Of note, the leukocyte antigen CD45 gene Ptprc was significantly increased and the ontology enrichment analysis revealed robust increase of immune response and inflammation in Ox1RΔSERT mice compared to control mice (Fig. 6e, f). The top 15 enriched gene ontology (GO) terms include positive regulation of cytokine production, myeloid leukocyte activation, regulation of inflammatory response, etc. (Fig. 6f). Among the significantly regulated genes, numerous relate to immune system, such as Cdl5, Fcgr1,
Table 1). Protein expression analysis supported the and thermogenesis. In Ox1R impaired mitochondrial dynamics, oxidative phosphorylation, and neurons in LH or orexinergic combination in orexin neurons (OrexinChR2-EYFP). Control mice were yellow fluorescent protein (EYFP) induced by Cre-loxP recombinase in orexin A-positive cells in LH (Supplementary Fig. 7e, k). In Ox2RΔSERT mice, the protein expression of Acox3, Mfn1, and Mff genes mediating mitochondrial fusion or fission were not significantly changed (Fig. 7f, Supplementary Table 1). Mfn1 gene, which mediates mitochondrial fusion showed a tendency to be decreased in Ox1RASERT mice, while Mfn2, Fis1, and Mff genes mediating mitochondrial fusion or fission were not significantly changed (Fig. 7f, Supplementary Table 1). Protein expression analysis supported the findings of impaired mitochondrial dynamics, oxidative phosphorylation, and thermogenesis. In Ox1RASERT mice, the protein expression of UCP-1, OXPHOS complex I CI-NDUFB8, complex IV CIV-UCQRC2, complex V CV-ATP5A, fusion proteins optic atrophy type 1 (OPA1) and import receptor TOM20, complex III CIII-UQCRC2, complex V CV-ATP5A, fusion proteins optic atrophy type 1 (OPA1) and import receptor TOM20, complex III CIII-UQCRC2, complex V CV-ATP5A, which functions mitochondrial fusion 1 (FIS1) were significantly reduced and complex II CII-SDBH also showed a tendency to be decreased (Fig. 7g–l). The mitochondrial protein import receptor TOM20, complex III CIII-UQCRC2, complex V CV-ATP5A, fusion proteins optic atrophy type 1 (OPA1) and mitofusin 2 (MFN2), and fusion proteins dynamin-related protein 1 (DRP1) and mitochondrial fission factor (MFF) remained unchanged (Fig. 7g–l). In contrast to obese Ox1RASERT mice, obese Ox2RASERT mice showed unaltered BAT weight, morphology as detected with haematoxylin and eosin (H&E) staining, gene expression levels of Acox3, Vegfa, Pparaα1a, Cysc, Mfn1, Mfn2, Dnm1l, Fis1 and Mff, and protein expression levels of UCP1, compared to control mice (Fig. 7m–p, Supplementary Fig. 6b).

Glucose metabolism upon optogenetic stimulation of orexin neurons in LH or orexinergic fibers in RPa. Next, we investigated the impact of activation of the orexin system on glucose metabolism by insulin tolerance test (ITT) and glucose tolerance test (GTT), using an optogenetic approach. Orexin-Cre mice were created and the expression pattern of Cre was analyzed by crossing Orexin-Cre transgenic mice and channelrhodopsin-2 (ChR2)-tdTomato 0/0 mice. tdTomato was specifically expressed in orexin A-positive cells in LH (Supplementary Fig. 7e, k). In addition, we could detect a small cluster of tdTomato single-labeled cells below the lateral ventricle and around the 3rd ventricle and several scattered cells in other brain regions (Supplementary Fig. 7a–j). We injected an adenovirus-associated vector (pAAV-EFla-double floxed-hChR2-EYFP) into the LH of Orexin-Cre mice to obtain expression of ChR2 and enhanced yellow fluorescent protein (EYFP) induced by Cre-loxP recombination in orexin neurons (OrexinChR2-EYFP). Control mice were injected with pAAV-EFla-DIO-EYFP (OrexinEYFP). The efficiency and specificity of ChR2-EYFP and EYFP expression were evaluated with immunostaining of orexin and EYFP in LH. The EYFP signal could be localized in orexin-positive cells exclusively (Fig. 8a). 70.95% and 76.23% of orexin neurons were EYFP positive in OrexinEYFP and OrexinChR2-EYFP mice, respectively (Fig. 8b).

The optical fiber was implanted above LH in AAV-injected mice to allow optogenetic activation of orexin neurons (Fig. 8c, Supplementary Fig. 8a). Blue light (473 nm) laser illumination (20 mW, 20 Hz, 10 ms pulse, 10 s on in every 45 s), which started 30 min before glucose or insulin injection and lasted until the end of ITT and GTT experiments under random-fed or 6-h fasting conditions respectively, significantly impaired glucose tolerance 15 min after glucose injection (Fig. 8d) and insulin sensitivity revealed by two-way ANOVA (F (1, 80) = 7.00, p = 0.0098; Fig. 8e). Accordingly, c-Fos expression was significantly increased in orexin neurons of OrexinChR2-EYFP mice compared to OrexinEYFP mice (fasted for 6 h) after 1 h of laser illumination (Fig. 8f, g). c-Fos positive orexin neurons were detectable throughout the LH and we did not observe topographic segregation. DR- and RPa-projecting cells were distinct and intermingled in LH, as revealed by retrograde tracing with red and green fluorophores injected to DR and RPa respectively (Fig. 8h–j).

Innervation of orexinergic nerve fibers was analyzed by staining of serotonin, orexin, and EYFP in RN of AAV-injected mice. There were abundant orexin- and EYFP-positive nerve fibers which co-localized with serotonin neurons in all RN regions, including DRD, DRV, MRD, MRV, and RPa, of control (Fig. 9a) and OrexinChR2-EYFP mice (Fig. 9b). This indicates that orexin nerve fibers innervate RN and the ChR2 protein travels successfully to these nerve endings. Further, we implanted the optical fiber above RPa in AAV-injected mice to selectively activate orexinergic fibers in this projection field (Fig. 9c, Supplementary Fig. 8b). Laser illumination (10 mW, 20 Hz, 10 ms pulse, 10 s on in every 45 s) in RPa, which started 30 min before glucose or insulin injection and lasted until the end of experiments, significantly improved glucose tolerance 15 min after glucose injection in GTT under 6-h fasting conditions (Fig. 9d). Insulin sensitivity remained unchanged upon laser illumination in RPa, as assessed by ITT (Fig. 9c). c-Fos positive serotonergic neurons in RPa were significantly increased in OrexinChR2-EYFP mice compared to OrexinEYFP mice (fasted for 6 h) after 1 h of laser illumination (Fig. 9f, g), without evidence for significant back-propagation to orexin neurons in the LH (Supplementary Fig. 9e, f).

Laser illumination in LH or RPa failed to affect glucose metabolism in control mice, as measured by ITT and GTT (Supplementary Fig. 9a–d).

Discussion

DR and MR in the midbrain and pons contain the majority of serotonergic neurons, which mainly project to the forebrain, while serotonergic neurons in caudal RN, such as RPa, mainly project to the brain stem and periphery. We find that, among the different RN regions, Ox1R is dominantly expressed in DRD while Ox2R is dominantly expressed in DRV, and only Ox1R could be clearly detected in serotonergic neurons in MRV and RPa. In line with this, a previously published RNA-seq data study shows that Ox2R is dominantly expressed in serotonergic neurons in DR, less expressed in the relatively dorsal and median regions of MR, and rarely expressed in ventral MR or caudal RN. In addition, a recently published scRNA-seq data study shows that Ox1R is expressed at different levels among 5 subtypes of serotonergic neurons in DR.

Both Ox1R and Ox2R mediate an excitatory direct response upon orexin stimulation in serotonergic neurons, as found in our study and previous studies by others. Consistent with the
**Fig. 7** Increased fat and impaired mitochondrial function in brown adipose tissue (BAT) of Ox1R−/− mice while BAT morphology and mitochondrial function are unaltered in Ox2R−/− mice fed a high-fat diet (HFD). **a** H&E staining of BAT of control or Ox1R−/− mice. HFD-Ctrl, n = 8; HFD-Ox1R−/−, n = 10. **b** Representative electronic microscope (EM) images of BAT of control and Ox1R−/− mice, and quantification of **c** mitochondrial area and **d** mitochondrial aspect ratio. n = 5, p = 0.033. **e** Relative mitochondrial DNA content of control and Ox1R−/− mice, n = 6. **f** Gene expression levels in BAT of control and Ox1R−/− mice. Control: n = 6 (Mfn1), 7 (Mfn2), 8 (Vegfa, Cycs, Mfn1, Dnm1l), and 9 (Acox3, Pparc1a, Fis1); Ox1R−/−, n = 11, p = 0.0026 (Acox3), 0.0031 (Vegfa), 0.0038 (Pparc1a), 0.024 (Cycs), 0.075 (Mfn1) and 0.0035 (Dnm1l). **g** Western blot images and quantification of UCP-1 and TOM20 protein. **h** J OPXHOS protein, and **i** mitochondrial fusion and fission protein in BAT of control and Ox1R−/− mice. UCP1 and FIS1 were from the same western blot gel and thus shared the loading control. HFD-Ctrl, n = 8; HFD-Ox1R−/−, n = 9, p = 0.020 (UCP-1), 0.015 (CI-NDUFB8), 0.094 (CII-SDH), 0.021 (CIV-MTCO1), 0.017 (Mfn1) and 0.0039 (Fis1). **m** H&E staining of BAT of control and Ox2R−/− mice. HFD-Ctrl, n = 11; HFD-Ox2R−/−, n = 9. **n** Gene expression levels in BAT of control and Ox2R−/− mice. HFD-Ctrl, n = 8 except for Cycs (n = 7); HFD-Ox2R−/−, n = 9 except for Cycs (n = 8). **o** Western blot images and quantification of UCP-1 protein in BAT of control and Ox2R−/− mice. HFD-Ctrl, n = 7; HFD-Ox2R−/−, n = 9. Scale bar: 100 mm in (a, m) and 2 μm (b). Data are represented as means ± SEM. *p < 0.05, **p < 0.01; as determined by unpaired two-tailed Student’s t-test. Source data are provided as a Source Data file.
Fig. 8 Optogenetic stimulation of orexin neurons impairs glucose tolerance. a Representative images of immunostaining of orexin and EYFP in the lateral hypothalamus (LH) of Orexin-Cre mice injected with Cre-dependent adeno-associated virus pAAV-EYFP (OrexinEYFP) or pAAV-ChR2-EYFP (OrexinChR2-EYFP). n = 3. b Quantification of percentages of GFP positive neurons in orexin neurons of mice injected with AAV. n = 3. Magenta, orexin; green, EYFP. c Schematic drawing of the strategy of optogenetic stimulating of orexin neurons. d Glucose tolerance test and e insulin tolerance test of OrexinChR2-EYFP mice with (laser on) or without (laser off) laser illumination in LH. d n = 10, p = 0.0056 (15 min); e n = 9, p = 0.081 (60 min). f Representative images of RNAscope in situ hybridizations of c-Fos and orexin in LH and g the quantification of percentages of c-Fos positive neurons in orexin neurons after laser illumination. n = 5 or 4, p = 0.027. Red, orexin; yellow, c-Fos. h Representative images of retrobeads injected in dorsal raphe nucleus (red beads) and raphe pallidus (green beads). i Representative images of retrobeads traveled to LH, in which the squares-indicated regions were amplified in (j). n = 5. Magenta, red beads; cyan, green beads. Scale bar: 200 μm (a, f), 100 μm (i) or 50 μm (h, j). Data are represented as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; as determined by unpaired two-tailed Student’s t-test (g) or two-way ANOVA followed by Sidak’s post hoc test (d, e). Two-way ANOVA revealed a significant main effect of genotype in (d) (F (1, 108) = 15.75, p = 0.0001), and (e) (F (1, 80) = 10.21, p = 0.002). Source data are provided as a Source Data file.

differential expression pattern of orexin receptors in DR. Ca²⁺ imaging reveals that, in DRV, the unselective endogenous agonist orexin A-induced excitation of serotonergic neurons is mainly mediated by Ox2R. Both electrophysiological and GCaMP experiments find that the excitation of serotonergic neurons by orexin B, which is more selective for Ox2R activation, is attenuated by Ox2R deletion in serotonergic neurons but not by Ox1R deletion. Together with our RNAscope findings for Ox1R or Ox2R in serotonergic neurons of Ox1RΔSERT or Ox1RΔSERT mice, respectively, this suggests that our Cre-loxP recombination-mediated conditional knockout model is specific and efficient.

Deletion of either orexin receptor in serotonergic cells is insufficient to alter glucose metabolism and/or energy homeostasis in lean mice. However, it has opposing effects under conditions of HFD-induced obesity when the orexin system is more activated. This indicates that orexin signaling in serotonergic neurons is more crucial for maintaining energy homeostasis under obese conditions, compared to lean conditions. Consistent with this hypothesis, we found orexin neurons...
were more activated in HFD-fed mice compared to CD-fed mice under fasting conditions. Previous reports also show that orexin receptors regulate energy metabolism differently in lean and obese mice. For example, overexpression of orexin decreases BW of mice fed a low-fat-diet or HFD, but deletion of Ox2R only abolishes the effect in mice on HFD10.

Ox1R signaling in serotonergic cells protects against HFD-induced insulin resistance, due to changes in peripheral glucose uptake but not hepatic gluconeogenesis. Specifically, Ox1R signaling in serotonergic neurons activates BAT thermogenesis and glucose uptake. Ox1R deletion in serotonergic cells of obese mice resulted in larger lipid droplets in BAT and increased BAT weight. BAT whitening attracts immune cells and tissue chronic inflammation, which further increases insulin resistance, under obese conditions39–42. Mitochondria are in an equilibrium of fusion and fission, and the mitochondrial elongation/fusion facilitates oxidative phosphorylation that produces ATP from glucose43–45. We find impaired mitochondrial function, including markers of reduced mitochondrial fusion, oxidative phosphorylation, lipid metabolism, and thermogenesis in BAT of obese mice that lack Ox1R in serotonergic neurons, suggesting that the capability of BAT to utilize glucose and fatty acids for thermogenesis and ATP production are both reduced. In line with this, BAT glucose uptake under steady-state conditions in hyperinsulinemic-euglycemic clamp studies is decreased in obese Ox1RΔSERT mice. Previous studies reported that BAT thermogenesis is activated by both the serotonin and the orexin systems. Orexin knockout mice display impaired thermogenesis due to the inability of brown preadipocytes to differentiate46. Another study fails to find detectable abnormalities in BAT development of orexin-deficient mice, and predicts that orexin controls BAT thermogenesis via a central pathway, based on the data that Ox1R is expressed low and Ox2R is undetectable in BAT15.

Serotonergic neurons in the rostral RPa play a key role in the control of BAT thermogenesis26. We find abundant orexinergic fibers innervating RPa, and optogenetic activation of these orexinergic fibers improves glucose tolerance. Ox1R but not Ox2R is expressed in serotonergic neurons in RPa, and consistent...
with this, BAT morphology and thermogenic factors remained unchanged when Ox2R is specifically deleted in serotonergic neurons. Therefore, our data suggest that Ox1R in serotonergic neurons in RPa is crucial to mediate the activation of BAT thermogenesis by orexin and serotonin systems, and thus to improve glucose metabolism.

Glucose uptake in skeletal muscle is also reduced in serotonergic-specific Ox1R-deleted obese mice. A previous study reported that orexin injection in the ventromedial hypothalamus stimulates skeletal muscle glucose uptake via activation of the sympathetic nervous system\(^\text{17}\). Indeed, orexin and serotonin systems both regulate the autonomic system\(^\text{48,69}\). However, further investigations are clearly needed to understand the mechanisms of Ox1R signaling in serotonergic neurons in the stimulation of skeletal muscle glucose uptake.

In contrast to Ox1R, Ox2R signaling in serotonergic neurons impairs glucose tolerance and contributes to insulin resistance induced by HFD feeding. The dominant Ox2R signaling in DRV could be important in glucose intolerance and insulin resistance upon HFD feeding. The impairment of glucose tolerance and insulin sensitivity by Ox2R in serotonergic neurons is mainly due to the impaired insulin-stimulated suppression of gluconeogenesis in liver. Although orexin deleted mice exhibit late-onset obesity, acute i.c.v. injection of orexin increases blood glucose levels\(^\text{14}\). In line with this, we find that optogenetic activation of orexin neurons in LH induces significant impairment of glucose tolerance and a trend toward impairment of insulin sensitivity by GTT and ITT. Central application of orexin increases the expression of G6Pase and Pepck in liver and the increase of Pepck is abolished by the deficiency of Ox2R but not Ox1R, suggesting that Ox2R mainly mediates the orexin-induced increase of gluconeogenesis in liver\(^\text{14}\). This supports our hypothesis that Ox2R signaling in serotonergic neurons, especially in DRV, potentially plays an important role in orexin-induced glucose elevation via increasing liver gluconeogenesis.

The orexin system shows a circadian rhythm and is also changed according to the nutrition state, which is related to its bidirectional regulation of blood glucose\(^\text{12,14}\). The differential roles of Ox1R and Ox2R in serotonergic neurons could partially explain the underlying mechanisms of this bidirectional regulation. Obese Ox1R\(^\text{ASERT}\) and Ox2R\(^\text{ASERT}\) mice exhibit opposite changes in GIR in euglycemic-hyperinsulinemic clamp experiments, for which mice are fasted for 4 h in the morning. Obese Ox1R\(^\text{ASERT}\) mice show impaired glucose tolerance in GTT after a 6-h (in the morning) but not 16-h (overnight) fasting period, while obese Ox2R\(^\text{ASERT}\) mice exhibit a more significant improvement in glucose tolerance in GTT after a 16-h fasting period compared to 6-h fasting. Ox1R and Ox2R signaling in serotonergic cells seems to dominantly regulate glucose metabolism under a shorter fasting condition at light phase or after a longer fasting time at dark phase, respectively. On the other hand, conditional knockout of orexin receptors in serotonergic neurons of obese mice significantly alters glucose tolerance during a GTT and alters glucose uptake and gene/protein expression levels in peripheral tissue and insulin sensitivity during euglycemic-hyperinsulinemic clamp experiments under fasting conditions, while insulin tolerance during ITT, BW and body composition under random-fed conditions remained unaltered. This indicates that orexin signaling in serotonergic neurons could be more relevant under nutrition-restricted conditions with respect to the control of glucose metabolism. Moreover, in random-fed obese Ox1R\(^\text{ASERT}\) mice (21-wk old), impaired BAT function was detected at molecular and morphological levels. These clear changes in BAT could be late-onset and the time of HFD exposure may not have been sufficient to translate these effects into significant changes in BW or body composition, while compensation of other peripheral organs is also possible. Clearly, further well-designed studies are needed to investigate the underlying mechanisms of the above findings.

Orexin acutely promotes feeding but in long term also promotes energy expenditure, in part by regulating spontaneous physical activity and supporting a sympathetic tone, which dominates in the control of energy balance. The serotonin system is also well known to be involved in the control of food intake and body weight\(^\text{24,25}\). However, in our study, deletion of orexin receptors in serotonergic neurons fails to alter locomotor activity or feeding behavior under random-fed conditions. This suggests that the regulatory role of orexin signaling in serotonergic neurons in glucose metabolism and energy homeostasis is independent of feeding or spontaneous physical activity.

We found intermingled and distinct LH cells projecting to DR or RPa. The boundaries of the heterogeneous RN subregions are not clear and their projections overlap, so it is important to define serotonergic neuron subtypes by both molecular and spatial specificity. Most strategies in previous reports about the differential functions of subregions of RN used micro-injections of drugs targeting the serotonergic system or analyzed serotonergic neuronal activity in experimental disease models\(^\text{30}\). The specific roles of subtypes of DR serotonergic neurons in energy homeostasis have been rarely studied so far. A recent study using viral-genetic methods shows that the subcortical-projecting serotonergic neurons preferentially localize in DRD and the cortical-projecting ones localized more in DRV. Further, they could show that the subcortical amygdala- and prefrontal-cortex-projecting DR neurons exhibit opposite responses to aversive stimuli and differentially regulate behaviors upon exposure to stress\(^\text{51}\). Our analysis reveals that the most differently expressed gene between Ox1R- and Ox2R-dominant serotonergic neurons is VGLUT3, and suggests more Ox2R-dominant serotonergic neurons are glutamatergic than Ox1R-dominant neurons. VGLUT3 has been shown to be higher expressed in serotonergic neurons in DRV compared to those in DRD, and it increases serotonin-1A receptor (5HT-1A)-mediated neurotransmission in RN and accelerates serotonin release at a specific subset of serotonergic nerve terminals\(^\text{51,52}\). Furthermore, DR neurons expressing vesicular transporters for gamma-aminobutyric acid (GABA), Vgat (Slc32a1), or for glutamate, VGLUT3, were shown to increase or suppress food intake, respectively\(^\text{53}\). According to scRNA-seq data, GABA receptors (Gabbr1 and Gabrg3) and VGLUT3 are enriched in different subtypes of serotonergic neurons, potentially suggesting that different subtypes of serotonergic neurons modulate food intake via distinct mechanisms\(^\text{50,53}\). Ox1R and Ox2R signaling relate to both the spatial and neurochemical heterogeneity of serotonergic neurons in DR. Together with our data, these reports support the hypothesis that serotonergic neurons in DRD and DRV have distinct roles in the modulation of glucose metabolism and energy homeostasis.

Only Ox1R but not Ox2R are expressed in serotonergic neurons in MRV. Interestingly, Ox2R is abundantly expressed in non-Sert-expressing neurons in MRV, which implicates the possibility of their crosstalk with Ox1R-expressing serotonergic neurons in the same area. Serotonergic neurons in DR and MR reciprocally connect to neurons in the hypothalamus (HTN), implying a functional role in regulating energy metabolism. The projections of DR and MR were mainly studied with neuron-subtype-unspecific tracing methods, and are considered more diverse than their inputs\(^\text{54,55}\). Distinct roles for MR and DR serotonergic neurons have been described and some researchers even suggest antagonism between them\(^\text{56,57}\). It will be interesting to elucidate the functional roles of orexin receptor signaling in this region in future studies.

Collectively, our study reveals differential expression patterns and functional roles of Ox1R and Ox2R signaling in serotonergic
neurons in the control of peripheral glucose metabolism, BAT thermogenesis, and liver gluconeogenesis. Further detailed studies to understand this complex neuronal network and underlying cellular mechanisms will help to develop possible therapies targeting the orexin or serotonin systems for obesity.

Methods
Animal care. All animal procedures were conducted in compliance with protocols approved by the local government authorities (Bezirksregierung Cologne, Germany) and were in accordance with National Institutes of Health guidelines. Permission to maintain and breed mice as well as all for experimental protocols in this study was issued by the Department for Environment and Consumer Protection - Veterinary Section, Cologne, North Rhine-Westphalia, Germany (84-02.04.2013.A335). Mice were housed in groups of 3–5 at 22 °C–24 °C using a 12-h light/12-h dark cycle, with humidity of 50–70%. Animals had ad libitum access to water and food at all times, and food was only withdrawn if required for an experiment. Animals were fed a NCD (sniff® R/M-H Phytoestrogenarm), which contains 57 KJ% carbohydrates, 34 KJ% from proteins, and 9 KJ% from fat, a CD (sniff® EF D12450B *mod. LS), which contains 67 KJ% carbohydrates, 20 KJ% from proteins and 13 KJ% from fat, or a HFD (sniff® FF acc. D12492 (f) mod.), which contains 21 KJ% carbohydrates, 19 KJ% from proteins and 60 KJ% from fat. BL/6 mice were purchased from Charles River, France. tdTomato fl/fl mice were purchased from The Jackson Laboratory (B6;129 S6- Gt(Rosa)26Stomt2(CAG-COP4-td2R2H14)Aijt, stock No: 012567).

Generation of Ox1R fl/fl mice and Ox2r fl/fl mice. Ox1R BAC (5′-AAGCTTACAGCAATGACCTGAGA-3′) and Ox2r BAC (5′-AGGAGCAGAGAAGGAGAAGATA-3′) were obtained from the Mouse Resource & Research Center supported by NIH (stock number: 017260-UCD) Chr2 tdTomato fl/fl mice were obtained from The Jackson Laboratory (B6; 129S-Gt(Rosa)26Stomt2(CAG-COP4-td2R2H14)Aijt, stock No: 012567).

Generation of Ox1R fl/fl mice and Ox2r fl/fl mice. Ox1R flox (BAC61, followed by temperature-induced recombineering. A PCR amplicon containing the IRES-Cre-FRT-NEO-FRT cassette) and the respective probes, according to the manufacturer’s instructions. Gapdh was used as the internal control. Relative expression was determined using a comparative method (2−ΔΔCT). Probes purchased from Thermofisher Scientific are shown in Supplementary Table 1.

Analysis of gene expression. BAT was dissected from 21-week-old male HFD-fed mice. Total RNA from BAT was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen). cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels were then determined with real-time quantitative RT-PCR using TaqMan® Gene Expression Master Mix (ThermoFisher Scientific) and the respective probes, according to the manufacturer’s instructions. Gapdh was used as the internal control. Relative expression was determined using a comparative method (2−ΔΔCT). Probes purchased from Thermofisher Scientific are shown in Supplementary Table 1.

RNA sequencing of BAT. BAT was dissected at the end of hyperinsulinenic-euglycemic clamp experiments. The tissue was frozen at −80 °C until the RNA was extracted with DNeasy digestion using the RNeasy Lipid Tissue Mini Kit (Qiagen) and RNase-Free DNAse Set (Qiagen), following the manufacturer’s instructions. RNA integrity was assessed using the Agilent RNA 6000 Nano Kit (5067-1511, Agilent Technologies) and the Agilent 2100 Bioanalyzer. RNA libraries were prepared using the Illumina® TruSeq® mRNA stranded sample preparation Kit. Library preparation started with 1 μg total RNA. After poly-A selection (using poly-T oligo-attached magnetic beads), mRNA was purified and fragmented using divalent cations under elevated temperatures. The RNA fragments underwent reverse transcription using random primers. This is followed by second-strand cDNA synthesis with DNA Polymerase I and RNase H. After end repair and A-tailing, indexing adapters were ligated. The products were then purified and amplified (14 PCR cycles) to create the final cDNA libraries. After library validation and quantification (Agilent tape station), equimolar amounts of the libraries were pooled. The pool was quantified by using the Qubit KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced for 35 million reads using an Illumina NovaSeq6000 instrument and a PE100 sequencing protocol.

For the statistical analysis, we applied the community-curated nfcore rnaseq analysis pipeline version 1.4.43. The gene-level quantification was carried out using Salmon 0.14.144 using the reference genome GRCh38 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.20). The differential gene expression analysis was done using the DESeq2 1.26.0 package and yielded 265 significant (adjusted p-value < 0.05) differentially expressed genes with fold-change ≥ 2. A gene ontology term analysis of the 265 differentially expressed genes was carried out using the clusterProfiler 3.14.366 R package and yielded 530 differentially expressed GO-terms of class biological process. Applying the simplified method of clusterprofiler, which removes similar gene ontologies, yielded subsequently a list of 131 differentially expressed biological process terms. Figure 6f shows the top 15 differentially expressed terms of that list.

Western blotting. BAT was dissected from 21-week-old male mice on HFD and homogenized in RIPA buffer (Sigma-Aldrich) added with cOmplete™ Protease

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Inhibitor Cocktail (Roche), using a FastPrep instrument (MP Biomedicals) and bulk beads (Bertin Corp.). The protein samples were separated on 10% Criterion™ TGXTM Precast Midi Protein Gel (BioRad) and transferred with Trans-BlotR Turbo™ Medi PVDF Transfer pack (BioRad). After blocking with 5% non-fat milk or a blocking reagent (Roche) in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature (RT) for 1 h, the membranes were incubated overnight at 4 °C with antibodies raised against pSer473-Akt (1:1000, #4060, rabbit mAb, Cell Signaling Technology), G6Pase (1:250, sc-25840, rabbit pAb, Santa Cruz Biotechnology), UCP-1 (1:200, sc-6528, goat pAb, Santa Cruz Biotechnology), Tom20 (1:100, sc-17784, mouse mAb, Santa Cruz Biotechnology), OPA1 (1:1000, 612607, mouse mAb, Prosci), ACTR3 (1:1000, ab57602, mouse mAb, Abcam), DRP1 (1:1000, #8570, rabbit mAb, Cell Signaling Technology), MFF (1:2000, 17090-1-AP, rabbit pAb, Proteintech®), FSI1 (1:500, 10956-1-AP, rabbit pAb, Proteintech®) or complex I-V subunits in the respiratory chain (1:1000, Total OXPHOS Rodent WB Antibody Cocktail, containing 5 mouse mAbs, ab10143, Abcam). Calnexin (1:5000, 208,880, rabbit pAb, Calbiochem) and β-actin (1:5000, ab8226, mouse mAb, Abcam). MNF2 (1:1000, ab56889, mouse mAb, Abcam), DRP1 (1:1000, #8570, rabbit mAb, Cell Signaling Technology), chicken anti-GFP (1:1000, ab13970, chicken pAb, Abcam), rabbit anti-TPH2 (1:1000, #51124, rabbit mAb, Cell Signaling Technology) and/or rabbit anticathepsin D (1:400, rabbit pAb, Sigma-Aldrich), for overnight at RT, washed in PBS for 3 x 10 min, and incubated with secondary antibodies (1:500), including Alexa Fluor 594 donkey anti-goat, FITC donkey anti-chicken, Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 647 donkey anti-rabbit (a11058, sa 1-7200, a21206 and a31573, Invitrogen), for 1 h at RT. After washing, slides were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories). Slides were stained together at one time for each experiment to have identical conditions for comparable signals. Slides were stored at 4 °C until imaging. Images were obtained using confocal laser scanning microscope Leica TCS SP8 or manually analyzed with ImageJ (Fiji version 1.50d).

RNAseq fluorescent in situ hybridization in lateral hypothalamus and raphe nuclei. Brains from BL6 mice (14 weeks, fixed for 6 h) on CD/HFD, and brains from Serp1Tomato, OxIRERT1Tomato, and Ox2RERT1Tomato mice (13–18 weeks) on NCD were post-fixed for 20–22 h at RT and cut (20 μm). Slides were stored at −80 °C until staining. All reagents were purchased from Advanced Cell Diagnostics and the staining was performed with the RNAscope Multiplex Fluorescent v2 kit and RNAscope 4-Plex Ancillary Kit (Advanced Cell Diagnostics) according to the user manual. Probes for OxIR (Hertr1, 471561-C3, and Ox2R (Hertr2, 471551, ACX) were designed according to the luciferase region. The probes for tTomato (tTomato, 317041-C2, and C3), c-Fos (c-Fos, 316921-C4), TP2 (TP2, 318691), Pet1 (Feu, 413241-C2), SERT (Slc6a4, 315851-C2), VGLUT3 (Slc17a8, 431261-C2), and orexin (Hcrtr2, 490461-C2) were commercially available by ACD. In brief, slides were briefly washed in diethyl-pyrocarbonate (DEPC)-treated 1 × PBS for 15 min at 60 °C for 15 min. After dehydrating and drying, all slides were stored at 4 °C until imaging. Images were obtained with confocal laser scanning microscope Leica TCS SP8 and manually analyzed with ImageJ/Fiji (Fiji version 1.50d).

Food intake and locomotor activity. Male mice (20-week old) on NCD and HFD under random-fed conditions were analyzed. Food intake, locomotor activity, and indirect calorimetry measurements were made in a PhenoMaster System (TSE Systems) as previously described47.

Hypoxisinsulinemic-euglycemic clamp studies. Surgical implantation of catheters in the jugular vein was performed in 17-week-old HFD-fed male mice as previously described48. Mice recovered from surgery for ~1 week and only mice that regained at least 90% of pre-surgery bodyweight were included in further experiments. After fasting for 4 h, mice were placed into chambers, which allow them to move freely throughout the clamp experiment. After a bolus infusion (0.8 μCi of D3-[2-3H]glucose (PerkinElmer) tracer solution, the tracer was infused continuously for 30 min), washed again in water and hybridized with the mixture of probes in different channels for 2 h at 4 °C. After hybridization, the slides were washed in 0.2 × SSC and 0.5 × SSC for 30 min each at 60 °C. Slides were dehydrated and mounted with Pertex (Tissuesave). Slides were stored at 4 °C, and imaged with Zeiss Imager M2 microscope and the software Zen 2 (Carl Zeiss AG).

Electronic microscope images of BAT. BAT samples were isolated from HFD-fed male mice (12–15 weeks) and fixed in 2% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer (pH 7.2) for 4 h at 4 °C. Samples were rinsed in 0.1 M cacodylate buffer (pH 7.2), post-fixed with 1% OsO4 in 0.1 M cacodylate buffer (pH 7.2) for 3 h at 4 °C, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in epoxy resin. Semi-thin sections of 500 nm were cut, followed by cutting into ultrathin sections of 70 nm, with a diamond knife (Diatome, Biel, Switzerland) on an ultramicrotome (EM-UC6, Leica). Ultrathin sections were placed on a 100 mesh grid, contrasted with 1.5% uranyl acetate for 15 min at 37 °C, put in lead citrate for 4 min after washing, and dried after additional washing. Images were taken on a transmission electron microscope (Zeiss EM1099 and JEOL JEM 2100Plus) at RT. Mitochondrial size and aspect ratio were analyzed with ImageJ/Fiji (version 1.50d).

Immunostaining in lateral hypothalamus and raphe nuclei. Brains were post-fixed for 6 h at 4 °C, and cut (30 μm) with Leica CM3050 S Research Cryostat. Sections were incubated in 0.3% glycine for 10 min after washing in 0.1 M PBS for 2 × 10 min. After washing in PBS for another 10 min, sections were incubated in 0.03% SDS (in PBS) for 10 min before they got blocked with 3% donkey serum (in PBS, 0.25% Triton X-100) for 1 h at RT. Afterwards, they were incubated with primary antibodies, including goat anti-orexin A (1:250, sc-8070, goat pAb, Santa Cruz biotechnology), chicken anti-GFP (1:1000, ab13970, chicken pAb, Abcam), rabbit anti-TPH2 (1:1000, #51124, rabbit mAb, Cell Signaling Technology) and/or rabbit anticathepsin D (1:400, rabbit pAb, Sigma-Aldrich), for overnight at RT, washed in PBS for 3 x 10 min, and incubated with secondary antibodies (1:500), including Alexa Fluoro 594 donkey anti-goat, FITC donkey anti-chicken, Alexa Fluoro 488 donkey anti-rabbit, Alexa Fluor 647 donkey anti-rabbit (a11058, sa 1-7200, a21206 and a31573, Invitrogen), for 1 h at RT before washing in PBS for 3 x 10 min. Slides were mounted with VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories). Slides were stained together at one time for each experiment to have identical conditions for comparable signals. Slides were stored at 4 °C until imaging. Images were obtained using confocal laser scanning microscope Leica TCS SP8 and manually analyzed with ImageJ/Fiji (version 1.50d).
processed through ion-exchange chromatography columns (Poly-Prep Prefilled Chromatography Columns, AG1-X8 formate resin, 200–400 mesh dry, Bio-Rad) to separate KCl and CaCl₂ and phosphate (2D6GP). Washed vivo glucose uptake was measured with NaCl, NaAT, and skeletal muscle (nmol x 1 min⁻¹ x 1 mm⁻²) was calculated based on the accumulation of 2D6GP in the respective tissue and the disappearance rate of 2D6G from plasma as described previously. Serum human insulin concentrations at baseline levels and at the end of the clamp studies were measured with DRG Ultra Sensitive Insulin ELISA Kit (EIA-2337).

**Virus injection and optical fiber implantation.** 13-week-old male Orexin-Cre mice received 1 mg/ml of tramadol in drinking water 2 days before the surgeries. On the day of surgery, mice were anesthetized with isoflurane and a bolus of buprenorphine (0.1 mg/kg BW) was given (i.p.) to reduce pain. Brain regions were identified according to the atlas. After alignment of the brain in the stereotaxic surgery platform, 1–3 small holes were drilled in skull at specific coordinates. The channelrhodopsin-2 (ChR2) and control virus (pAAV-EF1a-doble-flexed-fluxChR2(H134R)-EYP-WPRE-HGHpA and pAAV-Ef1a-Dio-EYFP, ~1 x 10^3 GC/ml, 333 nl, Addgene) was injected into LH uniilaterally for optogenetic stimulation in LH or bilaterally for checking virus expression and optogenetic stimulation when the fibers were pulled in house with a heating system. The coordinates from Bregma were: anterior-posterior, AP: –1.7 mm; medial-lateral, ML: ±0.9 mm; dorsal-ventral, DV: –3.2 mm. The GCaMP6 virus (AAV1.Syn.Flex.GCaMP6s.WPRE.SV40, ~4 x 10^12 GC/ml, 333 nl, Addgene) was injected to DR unilaterally for that laser stayed off. There was 1 week of recovery between each experiment. All surgeries were performed with a sterile surgical procedure. Mice were anesthetized with iso-flurane (B506; AbbVie Deutschland GmbH and Co KG, Ludwigshafen, Germany) and decapitated. Coronal slices (280 µm) containing DR were immediately implanted to stimulate LH (AP: –4.6 mm, ML: 0 mm, DV: –3.0 mm) of 12-14 day-old pups (postnatal) with micropipettes pulled in house with a heating system. The laser power was 20 mW to stimulate orexin neurons in LH and 10 mW to 2.5 mW/mm² (https://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php) to stimulate neurons in BAT, and skeletal muscle (nmol × g⁻¹, AAV1.Syn.Flex.GCaMP6s.WPRE.SV40, ~4 x 10^12 GC/ml, 333 nl, Addgene) was injected into DR with a heating system. On the day of surgery, mice were anesthetized with iso-flurane (B506; AbbVie Deutschland GmbH and Co KG, Ludwigshafen, Germany) and decapitated. Coronal slices (280 µm) containing DR were further processed. Five days later, mice were perfused and brains were further processed.

**Retrograde tracing.** Pre- and post-surgery treatment of mice were the same as described above. After alignment of the brain in the stereotaxic surgery platform, two small holes were drilled in the skull for DR (AP: –4.6 mm, ML: 0 mm, DV: –3.0 mm) of 12-14 day-old pups (postnatal) with micropipettes pulled in house with a heating system. The laser power was 20 mW to stimulate orexin neurons in LH and 10 mW to 2.5 mW/mm² (https://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php) to stimulate neurons in BAT, and skeletal muscle (nmol × g⁻¹, AAV1.Syn.Flex.GCaMP6s.WPRE.SV40, ~4 x 10^12 GC/ml, 333 nl, Addgene) was injected into DR unilaterally for that laser stayed off. There was 1 week of recovery between each experiment.

**Optogenetic stimulation in vivo.** Three weeks after virus injection, mice were transferred to the experimental cages for 1 week of habituation. At least 2 days before the experiments, a patch cord was connected to the optical fiber to allow acclimation. On the day of the experiment, a new patch cord replaced the old one. The laser was turned on 30 min before the injection of insulin or glucose, and the TTG and DIO signal were acquired as described above. The blue light (473 nm: 10 µm pulse, 20 Hz) was turned on for 10 s in every 45 s, and lasted until the end of ITT and GTT. Laser power was 20 mW to stimulate orexin neurons in LH and 10 mW to stimulate orexinergic fibers in RPa. The irradiance in the targeted area was above 2.5 mW/mm². Amphotericin B (A8888; Sigma) was dissolved in dimethyl sulfoxide to a concentration of 200 µg·mL⁻¹ (DMSO; D8418, Sigma) and added to the internal solution. The extended concentration of amphotericin B was ~120–160 µg·mL⁻¹. Amphotericin solutions were prepared from undissolved (weight/weight samples) (stored at a -4 °C temperature and allowed to come to room temperature on the day of use) every day. The corrected data were recorded continuously, and experiments started after Rₛ had reached steady state (~15–20 min), and the action potential amplitude was stable. The orexin-induced ΔF/F was determined, based on the high K+ solution. The extended concentration of amphotericin B was ~120–160 µg·mL⁻¹. Amphotericin solutions were prepared from undissolved (weight/weight samples) (stored at a -4 °C temperature and allowed to come to room temperature on the day of use) every day.
The orexin effects were analyzed by comparing the action potential frequencies that were measured during 2 min intervals that were recorded before and at the end of the peptide applications. To analyze the orexin responsiveness, the neuron’s firing rate averaged from 10 s intervals was taken as one data point. To determine the mean firing rate and standard deviation, 12 data points were averaged. On the single-cell level, a neuron was considered orexin-responsive if the change in firing induced by orexin was three times larger than the standard deviation (SD)\(80,81\).

General statistical methods. If not stated otherwise, all values are expressed as the mean ± SEM (standard error of the mean). Statistical analyses were conducted using GraphPad Prism 8, unless stated otherwise. Datasets with only two independent groups were analyzed for statistical significance using an unpaired two-tailed Student’s t test, and paired two-tailed t-test was used when data were matched. Datasets subjected to two independent factors were analyzed using two-way ANOVA followed by Sidak’s post hoc test, if not stated otherwise. All p-values < 0.05 were considered significant \(^{*}p<0.05\), \(^{**}p<0.01\), and \(^{***}p<0.001\), \(^{****}p<0.0001\).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The RNA-Seq data generated in this study were deposited in the GEO database under accession code GSE168203. The source data underlying Figs. 1c–i, k–m, 2–h, 3b, e, 4b–r, 5a–t, 6a–c, 7–n, 8b, d, e, g, 9d, e and Supplementary Figs. 1i, n, o, p, r, 3c, 4a–h, 5a–t, 6a, 9a–d, f are provided as a Source Data file. Raw data that support the findings of this study are available from the corresponding author upon reasonable request. Publicly available datasets used in this study include GRCh38 database (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.2) and “scRNA-seq_huang2019” (https://doi.org/10.7910/DVN/Q5QCC8).

There are no restrictions on data availability. Source data are provided with this paper.

Code availability
The source code for analysing the RNA-Seq data presented in this study are deposited on GitHub with the repository bruening-lab/Sert-Ox1R (https://github.com/bruening-lab/Sert-Ox1R). There are no restrictions on code availability.

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Author contributions

XX, A.C.H., and J.C.B. conceived the project, designed the experiments, analyzed the data, and wrote the manuscript with input from the other authors. A.K. and F.T.W. designed the targeting strategy for conditional Ox1R flox mice and Ox2R flox mice and A.C.H. generated the mouse lines. B.B.L., T.E.S., and D.K. performed the Orexin-Cre mice. XX performed all the experiments apart from hyperinsulinemic-euglycemic clamp experiments (X.X., A.S., and J.C.B.) and the experiments in the mouse lines. B.B.L., T.E.S., and D.K. generated the Orexin-Cre mice. XX performed all the experiments including the hyperinsulinemic-euglycemic clamp experiments and the experiments with the mouse lines. B.B.L., T.E.S., and D.K. performed all the experiments. B.B.L., T.E.S., and D.K. performed the Orexin-Cre mice. XX performed the Orexin-Cre mice. XX performed the Orexin-Cre mice. XX performed the Orexin-Cre mice.

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Competing interests

The authors declare no competing interests.
