ORIGINAL RESEARCH

Downregulation of Orexin Receptor in Hypothalamic Paraventricular Nucleus Decreases Blood Pressure in Obese Zucker Rats

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***Background*—**Orexin and its receptors are critical regulating sympathetic vasomotor tone under physiological and pathophysiological conditions. Orexin receptor 1 (OXR1) is upregulated in the paraventricular nucleus (PVN) in the hypothalamus and contributes to increased sympathetic outflow in obese Zucker rats (OZRs). We hypothesized that silencing OXR1 expression in the PVN decreases heightened blood pressure and elevated sympathetic outflow in OZRs.

***Methods and Results*—**An adeno-associated virus (AAV) vector containing a short hairpin RNA (shRNA) targeting rat OXR1 was designed to silence OXR1 expression in the PVN. The AAV-OXR1-shRNA or scrambled shRNA was injected into the PVN in OZRs. The arterial blood pressure in free-moving OZRs was continuously monitored by using a telemetry approach. The firing activity of spinally projecting PVN neurons in rat brain slices was recorded 3 to 4 weeks after injection of viral vectors. The free-moving OZRs treated with AAV-OXR1-shRNA had markedly lower OXR1 expression and lower mean arterial blood pressure, heart rate, and ratio of low- to high-frequency components of heart rate variability compared with OZRs treated with scrambled shRNA. Furthermore, AAV-OXR1-shRNA treatment markedly reduced renal sympathetic nerve activity and attenuated sympathoexcitatory response induced by microinjection of orexin A into the PVN. In addition, treatment with AAV-OXR1-shRNA substantially decreased the basal firing activity of spinally projecting PVN neurons in OZRs and attenuated the excitatory effect of orexin A on the firing activity of these neurons.

***Conclusions*—**These data suggest that chronic downregulation of OXR1 expression in the PVN reduces sympathetic vasomotor tone in obesity-related hypertension. **(*J Am Heart Assoc*. 2019;8:e011434. DOI: 10.1161/JAHA.118.011434.)**

Key Words: orexin and orexin receptor • hypothalamus • obesity-related hypertension • sympathetic nervous activity

The prevalence of obesity has increased substantially. Obesity is an independent risk factor for many major diseases such as hypertension, coronary heart disease, stroke, type 2 diabetes mellitus, cancer, and chronic kidney disease.[1,2] In particular, obesity may contribute 65% to 78% of the risk for essential hypertension.[3] Additionally, both experimental animal models of obesity and overweight humans have elevated sympathetic outflow.[3,4] This enhanced sympathetic outflow is critically involved in the pathogenesis of obesity-induced hypertension.[5–7] The obese Zucker rat (OZR) is widely used as a model of obesity attributable to a lack of functional leptin receptors, which is found in some obese humans.[8,9] OZRs also have hyperphagia, insulin resistance, hyperinsulinemia, hyperlipidemia, and heightened sympathetic outflow.[10,11] However, the neural mechanism involved in overactivation of sympathetic nervous system in obesity-related hypertension remains unclear.

Although the brain regions involved in heightened sympathetic nerve activity in obesity-related hypertension have yet to be fully identified, the hypothalamus and hindbrain regions

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Clinical Perspective

What Is New?

- We found that chronic downregulation of orexin receptor 1 expression in the paraventricular nucleus reduced blood pressure in free-moving obese Zucker rats.
- Silencing orexin receptor 1 in the paraventricular nucleus decreased the activity of spinally projecting paraventricular nucleus neurons and reduced arterial blood pressure and sympathetic tone in obese Zucker rats.

What Are the Clinical Implications?

- This finding provides a rationale for development of novel therapeutics targeting orexin receptor 1 to treat obesity-related hypertension.

appear to play crucial roles in mediating enhanced sympathetic outflow.[4,12,13] Among these regions, the paraventricular nucleus (PVN) in the hypothalamus is crucial to regulation of sympathetic nerve activity and neuroendocrine functions.[14] The PVN presympathetic neurons provide major sources of excitatory drive of sympathetic outflow through projections to the premotor neurons in the rostral ventrolateral medulla and the sympathetic preganglionic neurons in the intermediolateral cell column in the spinal cord.[15] It has been shown that the activity of PVN presympathetic neurons is increased in both animal models of essential hypertension[16] and obesity-related hypertension.[13] Previous studies have shown that suppression of neuronal activity in the PVN with overexpression of an inwardly rectifying K^+ channel caused sustained hypotension in spontaneously hypertensive rats.[17]

Neurons that synthesize orexins, including orexin A and orexin B, are limitedly distributed in the lateral hypothalamus.[18] Orexin-containing axons and orexin receptors (OXRs), including OXR1 and OXR2, are widely distributed in many brain regions[19–25] and play important roles in the regulation of cardiovascular function, energy balance, sleep and wakefulness, and feeding behavior.[20,26] A previous study has shown that microinjection of orexin A into the PVN increases arterial blood pressure (ABP) and sympathetic outflow in OZRs.[13] Orexin A can stimulate various types of neurons in brain regions involved in different functions, such as the lateral hypothalamus,[27] the arcuate nucleus,[28] and the PVN.[13,29]

We recently found that OXR1 expression is upregulated in the PVN and contributes to heightened sympathetic vasomotor tone in OZRs.[13] In anesthetized OZRs, acutely blocking OXR1 in the PVN by its antagonist decreased ABP and sympathetic outflow.[13] We hypothesized that silencing OXR1 expression in the PVN reduces obesity-related hypertension in OZRs. Taking advantage of the gene-silencing approach, we microinjected an adeno-associated viral vector carrying a short hairpin RNA (shRNA) targeting OXR1 into the PVN to decrease blood pressure and sympathetic outflow in OZRs.

Methods

All supporting data are available within this article and its online supplementary files.

Animals

Male OZRs (13–15 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in the animal facility in the Department of Veterinary Medicine and Surgery at the University of Texas MD Anderson Cancer Center at a controlled temperature and under controlled lighting (25°C±1°C, 12-hour light-dark cycle) with free access to rodent feed and tap water. Seventy male OZRs used in this study included 22 untreated OZRs, 24 OZRs with shRNA treatment, and 24 OZRs treated with the scrambled shRNA. All animal protocols and experimental procedures were approved by the University of Texas MD Anderson Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the ethical use of animals (protocol number 020602143).

shRNA Design and Microinjection into the PVN

To silence OXR1 expression, a distinct shRNA sequence targeting rat OXR1 (5′-gatccGCTACTTCATTGTCAACCTGTTCA AGAGACAGGTTGACAATGAAGTAGTTTTTT ACGCGTa-3′) and a scrambled shRNA sequence (5′-gatccAGTACTGCTTACGATAC GGTTCAAGAGACCGTATCGTAAGCAGTACTTTTTTTACGCGTa-3′) was used. A U6 promoter was used to drive expression of OXR1 shRNA or scrambled shRNA followed by green fluorescent protein (GFP). These components were integrated into an adeno-associated virus (AAV2: viral vector, serotype 2). Construction and packaging of the viral vector were performed by Vector Biolabs (Malvern, PA). The AAV-OXR1-shRNA vector was packaged at a titer of 1×10^{13} genome copies/mL. The vector was microinjected into the PVNs in OZRs as described previously.[30,31] In brief, under isoflurane-induced (2%) anesthesia, the rats were randomizely assigned to receive either AAV-OX1R-shRNA or scrambled shRNA and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA), and the vector was delivered into the PVN through a 26-gauge needle with its tip targeting the PVN at the following stereotaxic coordinates: 1.8 to 2.1 mm caudal from the bregma, 0.5 mm lateral to the midline, and 7.3 to 7.6 mm deep from the surface of the brain. The scrambled shRNA or AAV-OX1R-shRNA (100 nL) was injected over a period of 3 minutes. The surgical wound was closed by suturing the muscle and skin, and the rats were allowed to recover in home cages.

Western blotting and immunocytochemical staining were performed to measure the OXR1 expression levels in the rats. Under anesthesia induced by 2% isoflurane, the rats were quickly decapitated, and their brains were quickly removed. Bilateral PVN samples were obtained via micropunching. The total proteins were extracted in a radioimmunoprecipitation assay buffer and quantified using a Bradford protein assay. An aliquot of 40 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride membranes. These isolated proteins were immunoblotted with antibodies against OXR1 (rabbit antirat OXR1; Alomone Labs, Jerusalem, Israel), OXR2 (rabbit anti-rat OXR2; Merck Millipore, Burlington, MA), and glyceraldehyde 3-phosphate dehydrogenase (glyceraldehyde 3-phosphate dehydrogenase, rabbit anti-rat glyceraldehyde 3-phosphate dehydrogenase; Abcam, Cambridge, UK). The band signals were enhanced using a chemiluminescence kit (Fisher Scientific, Hampton, NH). The density of the OXR1 and OXR2 protein bands was decreased according to the respective glyceraldehyde 3-phosphate dehydrogenase bands in the same sample. The mean value of the band density of OXR1 and OXR2 proteins in OZRs given scrambled shRNA or without viral vector treatment was considered to be 1.

Immunocytochemical staining of brain tissue sections was performed as described previously.[13] Rats were deeply anesthetized by sodium pentobarbital (60 mg/kg, intraperitoneal) and intracardially perfused with 4% paraformaldehyde. Coronal brain slices at a thickness of 30 μm containing the PVN were obtained. The slices were blocked for 1 hour with 5% goat serum and incubated overnight with a primary antibody against OXR1 (rabbit anti-OXR1; Alomone Labs) and then a secondary antibody (Alexa Fluor-594–conjugated goat antirabbit; Abcam). The sections were viewed under a confocal microscope, and areas of interest were photographed.

Blood Pressure Measurement With Telemetry Approach

The ABP in free-moving rats was measured using a Millar telemetry system (Houston, TX). After the rats were anesthetized with 2% isoflurane, a catheter of the telemetry transmitter was inserted into the aorta.[32] Then, the transmitter was implanted and fixed in the abdominal cavity with a suture to the peritoneum. The abdominal wound was closed in 2 layers with interrupted sutures. Afterward, the rats were housed singly, and buprenorphine and enrofloxacin were given to them for 3 days. Then, 7 days after the implantation surgery, the shRNA viral vectors were microinjected into the bilateral PVN of rats with telemetry transmitter implanted. The blood pressure signals from the transmitters in free-moving rats were monitored, and the data were recorded and analyzed using a data acquisition system (LabChart 7; ADInstruments, Colorado Springs, CO).

The heart rate variability (HRV) in the OZRs was analyzed using the HRV module in the LabChart 7 software program (ADInstruments) as described previously.[33,34] A 10-minute baseline period was also used to calculate the baseline HRV. To perform spectral analysis of HRV, normal pulse-to-pulse tachograms were linearly resampled and transformed into the frequency domain using Welch's method with 50% overlapping and 1024-point Hann windows.[34,35] The power of the low-frequency (LF; 0.0625-0.1875 Hz) and high-frequency (HF; 0.1875-0.5625 Hz) bands was then used to calculate the LF/HF ratio. The LF/HF ratio has been a widely used index of sympathovagal balance, with the HF component representing vagal activity and the LF component being more representative of sympathetic activity.[33,34]

ABP, Heart Rate, and Renal Sympathetic Nerve Activity Recording and PVN Microinjection

Three to 4 weeks after viral vector injection, rats were anesthetized via intraperitoneal injection of a mixture of α-chloralose (60–75 mg/kg) and urethane (800 mg/kg) and were mechanically ventilated with room air through a trachea cannula connected to a rodent ventilator (CWE, Ardmore, PA). The concentration of CO_2 in the expiration was monitored by a CO_2 analyzer (CapStar-100; CWE) and maintained at 4% to 5% by adjusting the respiratory rate (≈60 bpm) and tidal volume (≈2.5 mL) throughout the experiment. The ABP was recorded through a cannula inserted into the left femoral artery, and the heart rate (HR) was extracted from the pulsatile blood pressure wave. Also, a small branch of the left renal sympathetic nerve was isolated from the surrounding tissue through an incision in the left flank[13] and put on a platinum electrode to acquire the nerve discharge. The renal sympathetic nerve activity (RSNA) was amplified (gain, 20,000–30,000) and filtered (band-pass, 100–3000 Hz) using an alternating-current amplifier (model P511; Grass Instrument, West Warwick, RI) and monitored using an audio amplifier (Grass Instrument). The RSNA and ABP signals were recorded using a 1401-PLUS analog-to-digital and Spike2 system (Cambridge Electronic Design, Cambridge, UK). The background noise was determined as the signals recorded 5 minutes after the rats were euthanized with an overdose of sodium pentobarbital (200 mg/kg, intravenous injection) at the end of each experiment and subtracted from the integrated RSNA values during data analysis. The basal level of RSNA (with subtraction of background noise) was set as 100%, and the percent change in RSNA from the baseline value was calculated.[13,36]

PVN microinjections of agents were performed as described previously.[36] In brief, a glass pipette (tip diameter,

20–30 μm) was advanced into the PVN through a small hole drilled in the surface of the skull at the following stereotactic coordinates: 1.6 to 2.0 mm caudal to the bregma, 0.5 mm lateral to the midline, and 7.0 to 7.5 mm ventral to the dura.[36] The agents were pressure-ejected using a calibrated microinjection system, and the injection procedure was monitored using an operating microscope. The injection sites in the PVN were first verified by a depressor response to microinjection of 5 nmol γ-aminobutyric acid (20 nL, 250 mmol/L). The location of the pipette tip and diffusion of the injectant in the PVN were examined and confirmed histologically after each experiment by 5% rhodamine-labeled fluorescent microspheres (0.04 μm) included in the drug solutions. At the completion of the experiment, the rats' brains were removed, fixed, and sectioned to determine the injection sites and area of diffusion of fluorescent dye according to the Paxinos and Watson atlas. Rats in which the pipette tip was misplaced outside the PVN were excluded from the data analysis.

Whole-Cell Recording in Brain Slice

The spinally projecting PVN neurons were retrogradely labeled by FluoSpheres (Invitrogen, Carlsbad, CA) injected into the spinal cord 2 to 3 weeks after viral vector injection. In brief, rats were anesthetized using 2% isoflurane in oxygen, and the T2 to T4 levels of the spinal cord were exposed surgically via laminectomy. FluoSpheres (red, 0.04 μm) were bilaterally injected through a glass pipette (tip, 20–30 μm) into the intermediolateral region of the T2 to T4 levels of the spinal cord in 3 separate 50-nL injections on each side. After the injections, the rats were given prophylactic penicillin (60 mg/100 g daily for 3 days) and an analgesic (0.5 mg/kg buprenorphine every 12 hours for 2 days). The rats were allowed to recover for 3 to 5 days to permit the FluoSpheres to be retrogradely transported to the PVN.

Brain slices at the hypothalamus level were prepared from the FluoSphere-injected rats. The rats were anesthetized using 2% isoflurane and decapitated. Their brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in mmol/L): 124.0 NaCl, 3.0 KCl, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 1.4 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$, which was continuously gassed with a mixture of 95% O_2 and 5% CO_2. Coronal hypothalamic slices (300 μm) were sectioned and incubated in aCSF continuously gassed with a mixture of 95% O_2 and 5% CO_2 at 34°C for 1 hour before recording.

PVN sections were transferred from the incubation chamber into a recording chamber filled and perfused with aCSF (saturated with 95% O_2 and 5% CO_2) at a speed of 3 mL/min with the temperature kept at 34°C by an inline heater. Labeled spinally projecting PVN neurons were identified under an upright microscope equipped with epifluorescent illumination and differential interference contrast optics. The recording electrodes were pulled from borosilicate capillaries using a micropipette puller and filled with an internal solution containing the following (in mmol/L): 140.0 K-gluconate, 2.0 $MgCl_2$, 0.1 $CaCl_2$, 10.0 HEPES, 1.1 EGTA, 0.3 Na_2-GTP, and 2.0 Na_2-ATP adjusted to pH 7.25 with 1 mol/L solution of KOH, and 270 ot 290 mOsm. The resistance of these electrodes was 3 6 MΩ when they were filled with the internal solution. Whole-cell patch-clamp recording was performed to record the spontaneously firing activity of spinally projecting neurons. The electrical signals were processed using a MultiClamp 700B amplifier, filtered at 1 to 2 kHz, and digitized at 10 kHz using a Digidata 1440 digitizer (Molecular Devices, San Jose, CA). The liquid junction potential, defined as the voltage difference between the recording pipette solution and external solution, was corrected for all the recordings. Because the spontaneous firing rate was irregular, the spontaneous firing activity was averaged for a 3-minute period for baseline, drug application, and washout when the firing activity was stable under each condition.

Drugs

Orexin A and N-(2-methyl-6-benzoxazolyl)-N'-1,5-naphthyridin-4-yl urea (SB334867) were purchased from Tocris Bioscience (Bristol, United Kingdom). Orexin A was dissolved in deionized water to form stock solutions and diluted with aCSF solution to designated final concentrations. SB334867 was dissolved in dimethyl sulfoxide. The final DMSO concentration in bath solution was 0.05%, which had on effects on the firing activity of PVN neurons.

Data Analysis

The mean ABP was obtained by integrating the blood pressure waveform over the recording period. The baseline values of mean ABP, HR, and RSNA were obtained by averaging these variables over a period of 3 minutes immediately before each treatment. Response values after each intervention were averaged over 60 seconds when the maximal responses occurred. The firing activity was analyzed using the MiniAnalysis software program (version 6.0). The membrane potential of recorded neurons was determined between adjacent discharges when it was stable or the neuron was silent. These data were analyzed by staff who were blinded to group-assignment information by using the GraphPad Prism 7. For comparisons of values in 2 groups, the Student *t* test was performed by using the GraphPad Prism 7. For comparisons of values in 3 or more groups, repeated measures ANOVA with Dunnett's post hoc test or 1-way ANOVA with Tukey's post hoc test was performed to compare responses among groups. *P* levels <0.05 were considered significant.

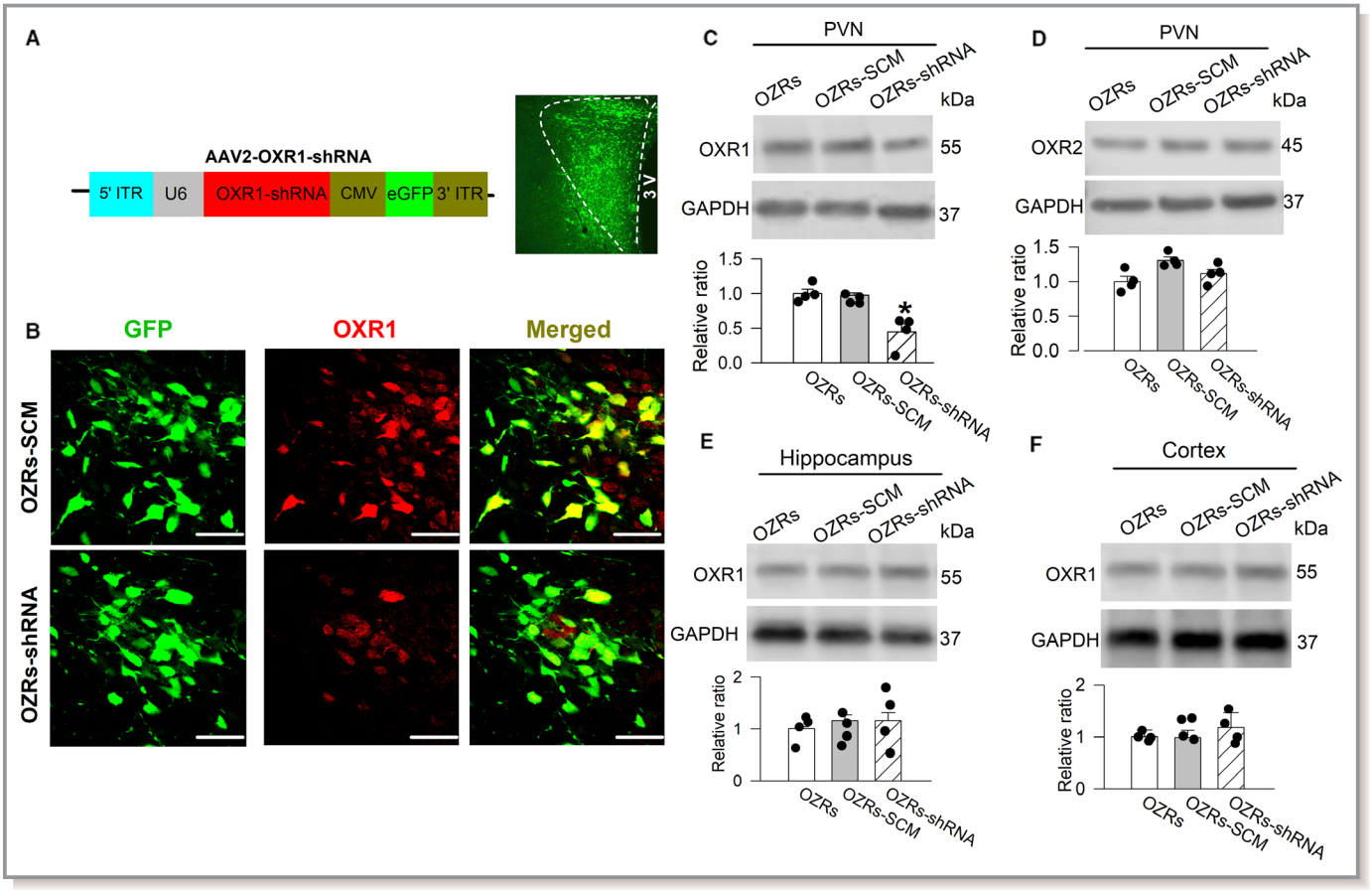

Figure 1. Delivery of AAV-OXR1-shRNA into the PVN induced robust downregulation of OXR1 expression in OZRs. **A**, Construction of AAV-OXR1-shRNA followed by GFP and the GFP expression in the PVN 3 to 4 weeks after microinjection of the vector into the PVN. **B**, Representative images showing GFP-labeled neurons (green) and OXR1 immunoreactivity-positive neurons (red) in the PVN 3 to 4 weeks after delivery of AAV-OXR1-shRNA or scrambled shRNA. **C** and **D**, Western blots of OXR1 and OXR2 expression in the PVN of untreated OZRs, OZRs treated with AAV-OXR1-shRNA, and OZRs treated with scrambled shRNA. Note that AAV-OXR1-shRNA treatment markedly decreased OXR1 expression without significantly affecting OXR2 expression in the PVN. **E** and **F**, Western blots of OXR1 expression in the hippocampus (**E**) and frontal cortex (**F**) in OZRs treated with AAV-OXR1-shRNA or scrambled shRNA. Data are presented as the mean±SEM. One-way ANOVA with Tukey's post hoc test was used to analyze differences in protein expression among the experimental groups. **P*<0.05 compared with the values in scrambled shRNA-treated OZRs. AAV indicates adeno-associated virus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; OXR1 orexin receptor 1; OZRs, obese Zucker rats; OZRs-SCM, OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; PVN, paraventricular nucleus of the hypothalamus; shRNA, short hairpin RNA.

Results

Verification of OXR1 Silencing in the PVN by Treatment With AAV-OXR1-shRNA

OXR1 expression was profoundly higher in the PVN in OZRs than in lean Zucker rats.[13] We used AAV-OXR1-shRNA to downregulate OXR1 expression in the PVN in OZRs. Specifically, we microinjected AAV-OXR1-shRNA into the PVN of OZRs (Figure 1A and 1B). To confirm that treatment with AAV-OXR1-shRNA downregulates OXR1 expression in the PVN, we immunostained hypothalamus tissue by using an antibody against OXR1 3 to 4 weeks after AAV-OXR1-shRNA injection. The majority of OXR1-positive PVN neurons were targeted by AAV-OXR1-shRNA or scrambled shRNA as indicated by GFP fluorescence. Compared with rats treated with scrambled shRNA, in which most of the GFP-tagged PVN neurons displayed OXR1 immunoreactivity, those treated with AAV-OXR1-shRNA largely had decreased OXR1 immunoreactivity in the PVN neurons (Figure 1B).

Western blot analysis revealed that OXR1 expression levels in OZRs treated with AAV-OXR1-shRNA were significantly lower than those in scrambled shRNA-treated or untreated OZRs (n=4 rats/group; *P*=0.0014 for OZRs treated with AAV-OXR1-shRNA versus the scrambled group, *P*=0.0040 for OZRs treated with AAV-OXR1-shRNA versus the untreated group) (Figure 1C). Both OXR1 and OXR2 are expressed in the PVN[21,37]; thus, we also determined whether treatment with

AAV-OXR1-shRNA affected OXR2 expression levels in the PVN. OXR2 expression levels in OZRs treated with AAV-OXR1-shRNA did not differ significantly from those OZRs treated with scrambled shRNA and untreated OZRs and (Figure 1D). To rule out the possibility that AAV-OXR1-shRNA in the PVN may affect OXR1 expression levels in other hypothalamus subregions, we measured the OXR1 expression in the ventromedial hypothalamus and dorsomedial hypothalamus located adjacently to the PVN. The expression of OXR1 in both the ventromedial hypothalamus and the dorsomedial hypothalamus did not differ among untreated OZRs, OZRs treated with scrambled shRNA, and OZRs treated with OXR1-shRNA (Figure S1). We also measured OXR1 protein levels in the hippocampus and the frontal cortex. The OXR1 levels were not different among untreated OZRs, OZRs treated with scrambled shRNA, and OZRs treated with OXR1-shRNA (Figure 1E and 1F).

Silencing of PVN OXR1 Expression Reduces ABP in Free-Moving OZRs

Acutely blocking OXR1 in the PVN by its antagonist decreased ABP and sympathetic outflow in anesthetized OZRs.[13] We sought to determine the effect of silencing of OXR1 expression in the PVN on ABP in OZRs. We monitored ABP in conscious, free-moving OZRs using a telemetric system. Microinjection of scrambled shRNA into the PVN did not alter the ABP 6 weeks after the microinjection. However, in OZRs treated with AAV-OXR1-shRNA, ABP started to decrease 3 weeks after microinjection of the viral vectors and produced peak depressor response on the 28th day after the injection (n=6, P=0.0011) (Figure 2A and 2B). In addition, HR was significantly decreased on the 28th day after AAV-OXR1-shRNA microinjection (n=6, P=0.0014) (Figure 2B). The ABP in OZRs treated with AAV-OXR1-shRNA decreased from

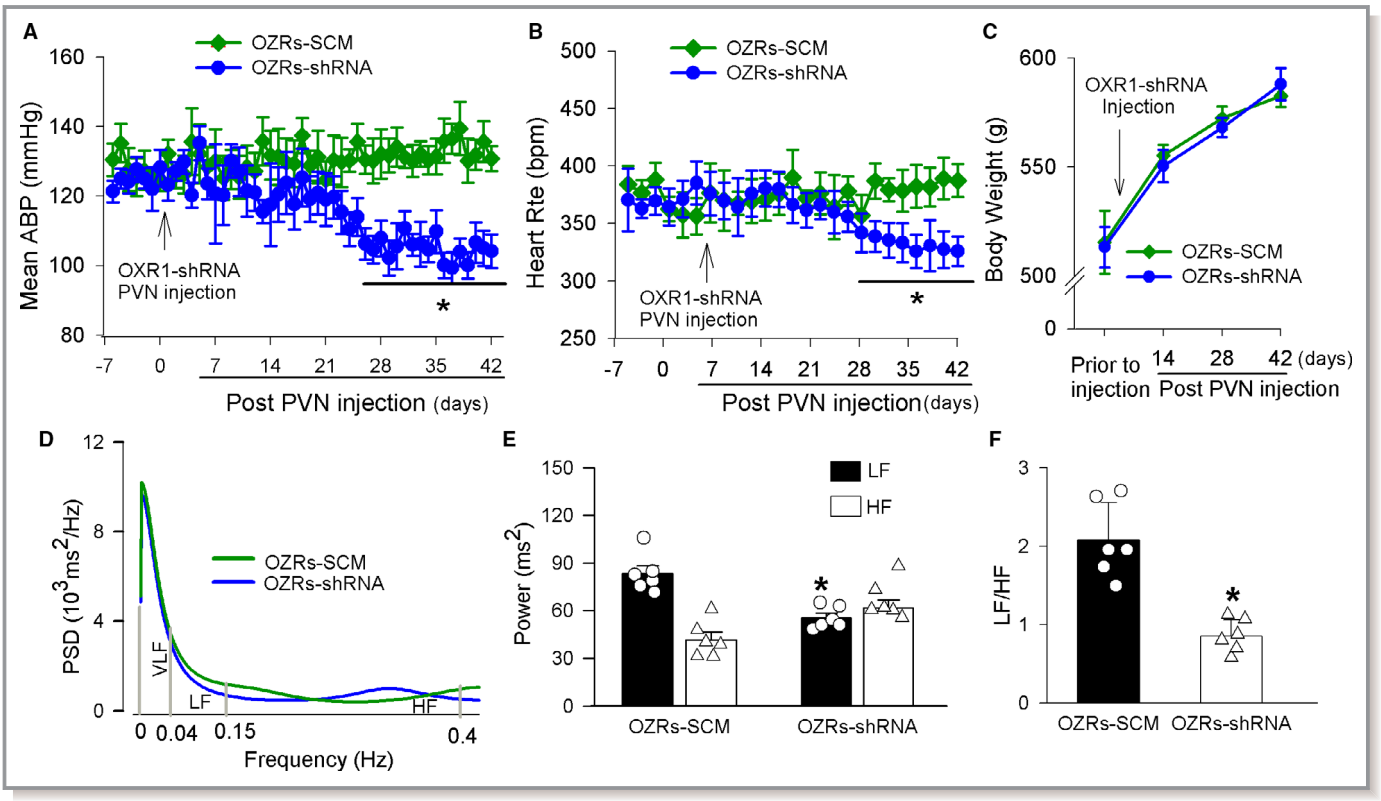

Figure 2. Silencing of OXR1 in the PVN by AAV-OXR1-shRNA decreased the mean ABP in OZRs. **A** and **B**, Time course of mean ABP (**A**) and HR (**B**) measured using telemetry in 6 free-moving OZRs with microinjections of AAV-OXR1-shRNA or scrambled shRNA (Scr-shRNA) into the PVN. The mean ABP and HR decreased considerably in OZRs 3 to 4 weeks after microinjection of AAV-OXR1-shRNA. **C**, Body weight changes in OZRs treated with AAV-OXR1-shRNA or scrambled shRNA. **D** through **F**, Spectral analysis of HR variability and summary data showing that treatment with AAV-OXR1-shRNA substantially decreased the LF components and the ratio of LF/HF when compared with treatment with scrambled shRNA (n=6 rats/group). Data are presented as the mean±SEM. One-way ANOVA with Tukey's post hoc test was used to analyze differences among experimental groups. *P<0.05 compared with the values in scrambled shRNA-treated OZRs. AAV indicates adeno-associated virus; ABP, arterial blood pressure; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; OXR1, orexin receptor 1; OZRs, obese Zucker rats; OZRs-SCM, OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; PVN, paraventricular nucleus of the hypothalamus; shRNA, short hairpin RNA.

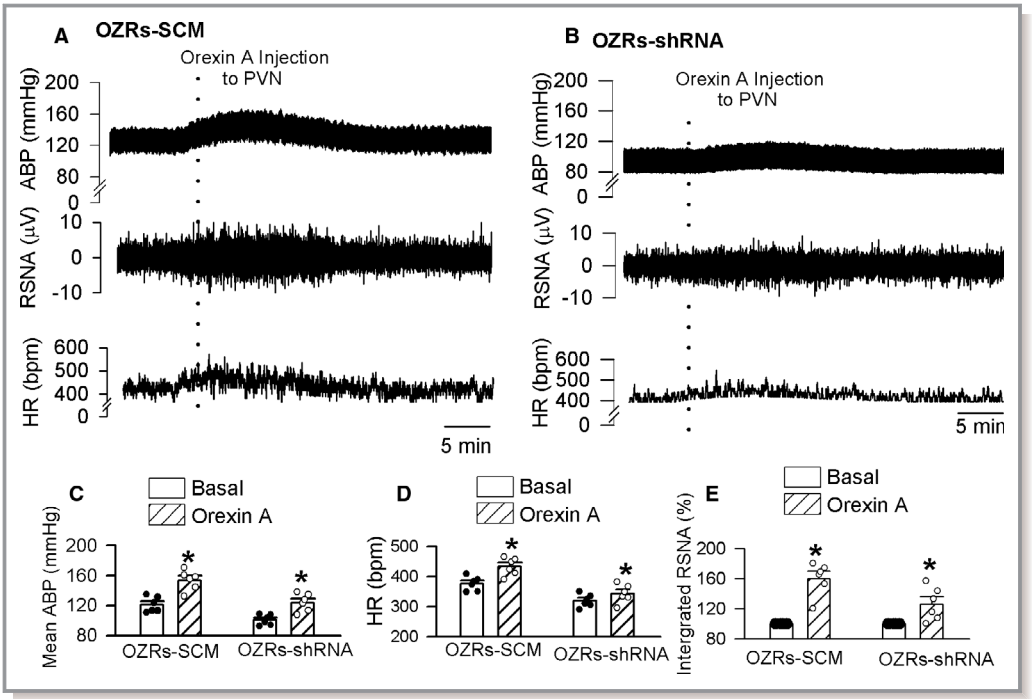

Figure 3. Treatment with AAV-OXR1-shRNA decreased ABP and sympathetic outflow in OZRs. **A** and **B**, Original traces showing that microinjection of AAV-OXR1-shRNA (OXR1-shRNA) (**B**) and scrambled shRNA (**A**) into the PVN decreased basal ABP, HR, and RSNA in OZRs. Furthermore, bilateral microinjection of orexin A (30 pmol in 50 nL of aCSF) into the PVN induced sympathoexcitatory response in OZRs treated with scrambled shRNA, an effect that was markedly attenuated in OZRs treated with AAV-OXR1-shRNA. **C** through **E**, Summary data showing the responses of mean ABP (**D**), HR (**D**), and RSNA (**E**) in OZRs treated with scrambled shRNA or AAV-OXR1-shRNA to bilateral injection of orexin A (30 pmol in 50 nL of aCSF) into the PVN (n=6 rats/group). Data are presented as the mean±SEM. One-way ANOVA with Tukey's post hoc test was used to analyze differences among experimental groups. **P*<0.05 compared with basal values in each group. #*P*<0.05 compared with respective values in OZRs treated with scrambled shRNA. 3V, third ventricle. AAV indicates adeno-associated virus; ABP, arterial blood pressure; HR, heart rate; OXR1, orexin receptor 1; OZRs, obese Zucker rats; OZRs-SCM, OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; PVN, paraventricular nucleus of the hypothalamus; RSNA, renal sympathetic nerve activity.

121.10±3.08 mm Hg to 104.12±4.78 mm Hg (*P*=0.0011), and the HR decreased from 370.61±27.21 bpm to 326.00±12.70 bpm 4 weeks after treatment with AAV-OXR1-shRNA (Figure 2A and 2B). Because high blood pressure is related to obesity in OZRs, we also sought to determine whether AAV-OXR1-shRNA microinjection into the PVN changes OZR body weight. We found that the body weights of OZRs treated with scrambled shRNA and those treated with AAV-OXR1-shRNA did not differ before and after viral vector injection into the PVN (Figure 2C).

We next analyzed the HRV to assess the autonomic nervous system activity in OZRs treated with scrambled shRNA or AAV-OXR1-shRNA. Analysis of the components of HRV is a widely used approach to assessing sympathovagal balance.[38] In the spectral analysis of HRV in OZRs treated with AAV-OXR1-shRNA or scrambled shRNA, we found that the LF domain was significantly lower but the HF domain was significantly higher in OZRs treated with AAV-OXR1-shRNA than in those treated with scrambled shRNA (n=6 rats/group; *P*=0.0006 for LF comparison, and *P*=0.0041 for LF comparison) (Figure 2D and 2E). Also, the LF/HF ratio was significantly lower in OZRs treated with AAV-OXR1-shRNA than in those treated with scrambled shRNA (n=6 rats/group; *P*=0.0002) (Figure 2F).

Silencing of OXR1 Expression in the PVN Decreases Basal Vasomotor Tone and Suppresses Sympathoexcitatory Effect of Orexin A in OZRs

The following experiment was performed by using rats 3 to 4 weeks after viral vector injection. The baseline ABP and HR were significantly lower in OZRs treated with AAV-OXR1-shRNA than in those treated with scrambled shRNA under anesthesia (n=6 rats/group; *P*=0.0024 for BP comparison, and *P*=0.0014 for HR comparison) (Figure 3A through 3D). Furthermore, microinjection of orexin A (30 pmol in 50 nL of aCSF)[13] into the

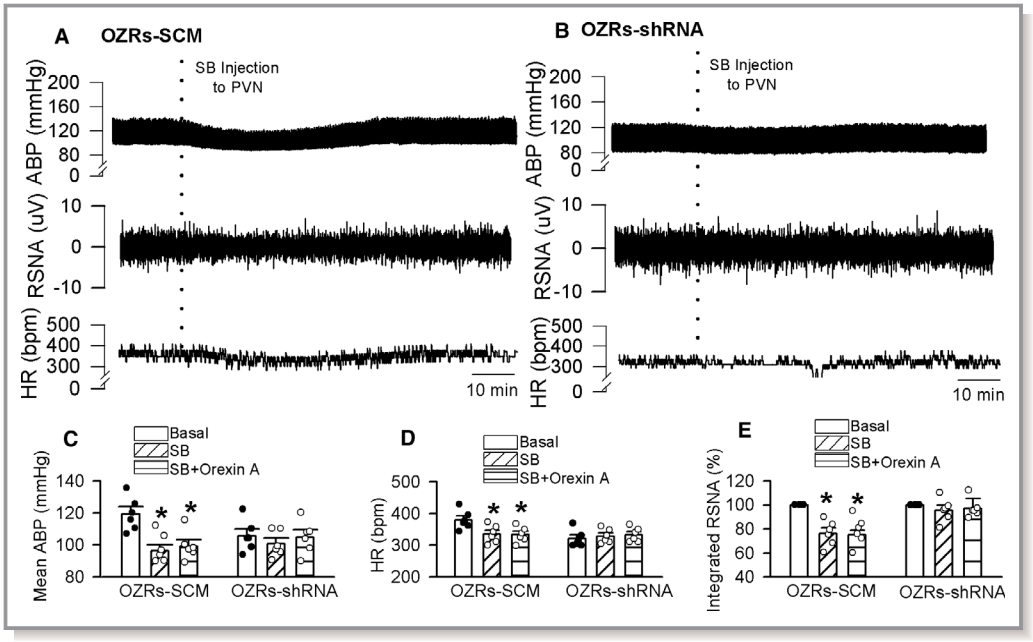

Figure 4. Silencing of OXR1 expression in the PVN eliminated the depressor response to blockade of OXR1 in the PVN in OZRs. **A** and **B**, Original traces of ABP, HR, and RSNA showing that bilateral microinjection of SB334867 (1 nmol in 50 nL of aCSF) into the PVN decreased the ABP, HR, and RSNA in OZRs treated with scrambled shRNA (**A**) but had no effect on these variables in OZRs treated with AAV-OXR1-shRNA (**B**). **C** through **E**, Summary data showing the mean ABP (**C**), HR (**D**), and RSNA (**E**) in OZRs treated with scrambled shRNA or AAV-OXR1-shRNA before and after microinjection of SB334867 into the PVN (n=6 rats/group) **P*<0.05 compared with the basal levels in each group. AAV indicates adeno-associated virus; ABP, arterial blood pressure; HR, heart rate; OXR1, orexin receptor 1; OZRs, obese Zucker rats; OZRs-SCM, OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; PVN, paraventricular nucleus of the hypothalamus; RSNA, renal sympathetic nerve activity; SB, SB334867; shRNA, short hairpin RNA.

PVN markedly increased the ABP, HR, and RSNA in OZRs treated with scrambled shRNA (Figure 3A and 3C through 3E). However, treatment with AAV-OXR1-shRNA largely attenuated the increases in ABP, HR, and RSNA induced by microinjection of orexin A into the PVN (Figure 3B and 3D through 3E). These data suggested that silencing of OXR1 expression in the PVN suppressed the sympathoexcitatory effect of orexin A.

We next determined whether OXR1 in the PVN mediated the depressor response and sympathoinhibitory effect induced by treatment with AAV-OXR1-shRNA in anesthetized rats. Microinjection of SB334867, a selective antagonist of OXR1 (1 nmol in 50 nL of aCSF), into the PVN decreased the ABP, HR, and RNSA in 6 OZRs treated with scrambled shRNA (*P*=0.0003) (Figure 4A and 4C through 4E). However, we did not observe a sympathoinhibitory effect of microinjection of SB334867 into the PVN in OZRs treated with AAV-OXR1-shRNA (n=6; *P*>0.05) (Figure 4B through 4E).

Silencing of OXR1 Expression Decreases the Firing Activity of Spinally Projecting PVN Neurons

The presympathetic PVN neurons provide excitatory drive to elevated sympathetic outflow in essential and obesity-related hypertension.[36,39] Thus, we determined whether treatment with AAV-OXR1-shRNA decreased the firing activity of spinally projecting PVN neurons in OZRs. The experiment was performed in rats 3 to 4 weeks after viral vector injection. We found that the baseline spontaneous firing activity of labeled PVN neurons was significantly lower in OZRs treated with AAV-OXR1-shRNA than in those treated with scrambled shRNA (n=12 neurons from 6 OZRs treated with AAV-OXR1-shRNA and n=11 neurons in 5 OZRs treated with scrambled shRNA; *P*=0.0001) (Figure 5). Bath application of orexin A (100 nmol/L) significantly increased the firing rate of labeled PVN neurons in OZRs treated with scrambled shRNA (n=12 neurons from 6 rats; *P*=0.0002). However, in OZRs treated with AAV-OXR1-shRNA, the excitatory effect of orexin A (100 nmol/L) on labeled PVN neurons was largely attenuated.

In another group of labeled PVN neurons from OZRs treated with scrambled shRNA, bath application of the OXR1 antagonist SB334867 (10 μmol/L) significantly decreased the spontaneous firing activity of labeled PVN neurons (n=10 neurons from 5 OZRs; *P*=0.0015) (Figure 6). However, this inhibitory effect of SB334867 was not observed in OZRs that received treatment with AAV-OXR1-shRNA (n=12 neurons from 6 rats).

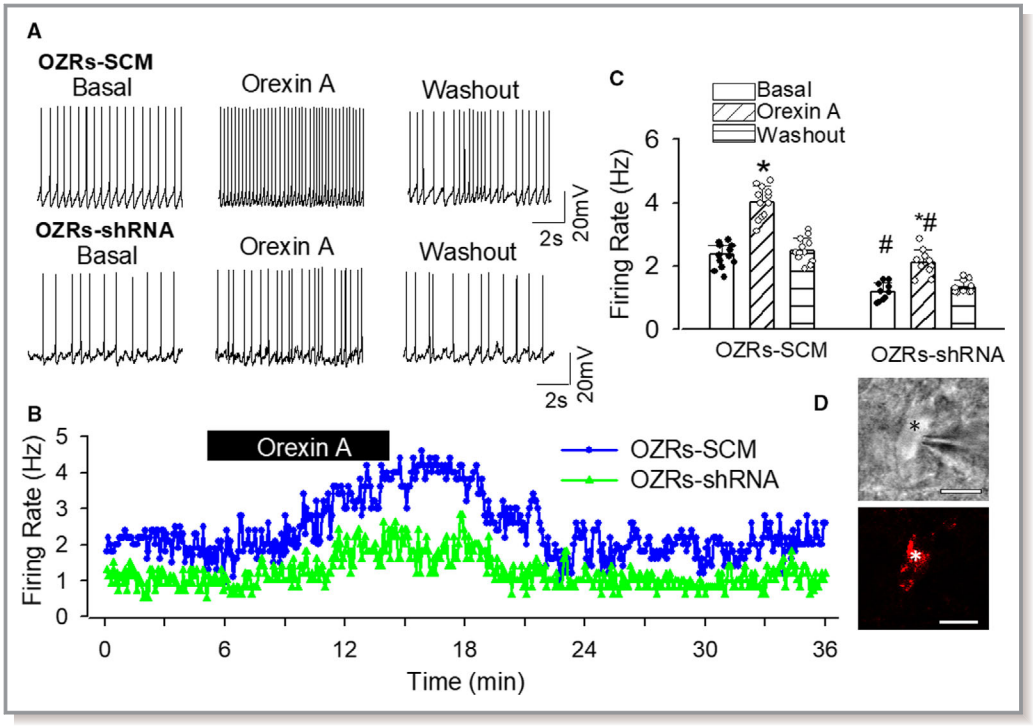

Figure 5. Silencing of OXR1 expression decreased the basal activity of PVN presympathetic neurons and eliminated the excitatory effect of orexin A on these neurons in OZRs. **A** and **B**, Representative raw tracings (**A**) and a frequency histogram (**B**) showing that treatment with AAV-OXR1-shRNA decreased the basal firing activity of spinally projecting PVN neurons. Bath application of orexin A (100 nmol/L) increased the firing rate of spinally projecting PVN neurons in OZRs treated with scrambled shRNA. This excitatory effect of orexin A was considerably attenuated in OZRs treated with AAV-OXR1-shRNA. (**C**) Summary data showing that treatment with AAV-OXR1-shRNA decreased the basal firing rate of spinally projecting PVN neurons and attenuated the excitatory effect of bath application of orexin A on spinally projecting PVN neurons (n=15 neurons in each group). **D**, A FluoSphere-labeled PVN neuron (*) in brain slice viewed with and infrared differential interference contrast optics (upper panel) and fluorescence illumination (lower panel). The recording electrode was marked with an arrow. The scale bars are 10 μm. $^{*}P<0.05$ compared with the basal values in each group; $^{\#}P<0.05$ compared with respective values in OZRs treated with scrambled shRNA. AAV indicates adeno-associated virus; OZRs, obese Zucker rats; OZRs-SCM, OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; shRNA, short hairpin RNA.

Discussion

In this study, we determined the chronic effect of downregulation of OXR1 expression in the PVN on ABP, sympathetic vasomotor tone, and presympathetic PVN neurons in rats with obesity-related hypertension. This study is the first to demonstrate that silencing OXR1 expression in the PVN causes sustained reduction of ABP in freely moving OZRs as well as decreased sympathetic outflow and excitability of presympathetic PVN neurons. The findings from the present study suggest that downregulation of OXR1 expression in the PVN is sufficient to significantly reduce elevated ABP and sympathetic vasomotor tone in obesity-related hypertension. The observed reduction of sympathetic outflow induced by OXR1 silencing was most likely due to inhibition of PVN presympathetic neurons.

To silence OXR1 expression, we designed a specific shRNA targeting OXR1 and cloned into AAV2 vector. Microinjection of this AAV-OXR1-shRNA into the PVN effectively downregulated OXR1 protein expression in the injection region. The downregulation of OXR1 expression induced by AAV-OXR1-shRNA was limited to the PVN region because OXR1 expression levels in the ventromedial hypothalamus, dorsomedial hypothalamus, hippocampus, and frontal cortex did not differ between rats treated with AAV-OXR1-shRNA and those treated with scrambled shRNA. Also, few GFP-positive neurons were found in surrounding regions such as dorsomedial hypothalamus and ventromedial hypothalamus, which are involved in the regulation of blood pressure. The PVN is a heterogeneous nucleus containing many types of neurons such as interneurons and projection neurons.[40] Thus, the neuronal promoter used in the AAV-OXR1-shRNA vector may not selectively downregulate OXR1 expression in PVN presympathetic neurons. The OXR2 expression level in the PVN was not altered by treatment with AAV-OXR1-shRNA,

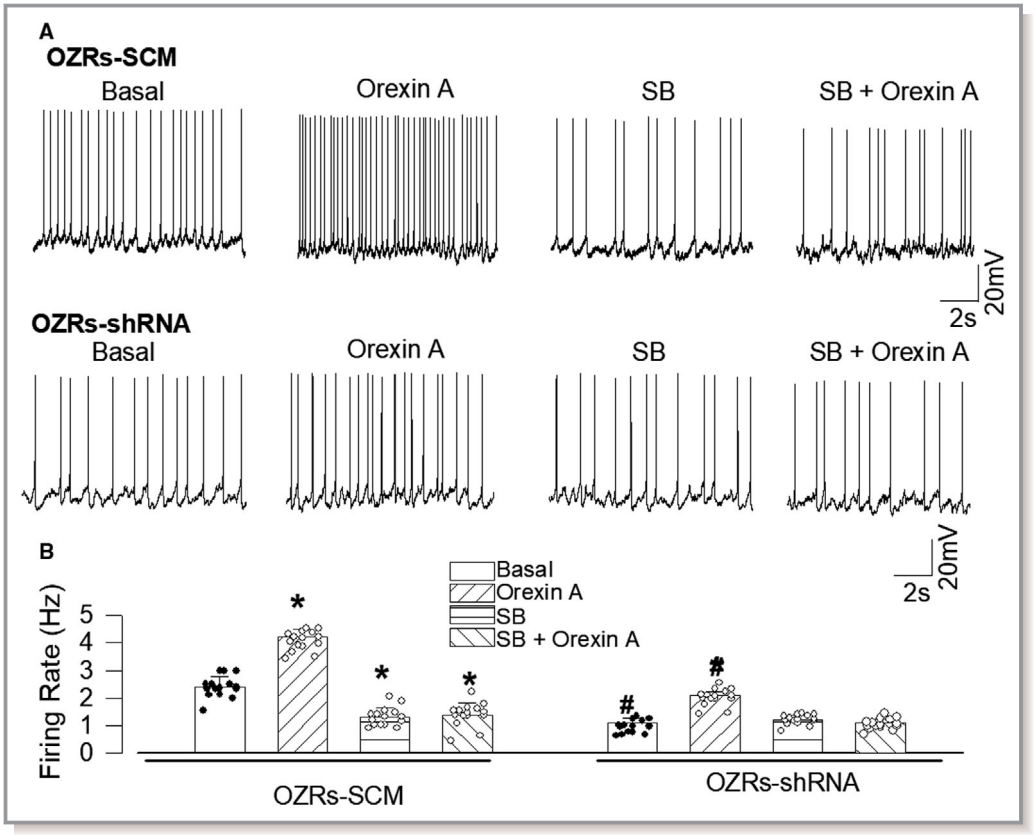

Figure 6. Silencing of OXR1 expression in the PVN eliminated the inhibitory effect of OXR1 blockade on spinally projecting PVN neurons in OZRs. **A** and **B**, Representative raw tracings (**A**) and summary data (**B**) showing that bath application of SB334867 (10 μmol/L) decreased the firing rate of spinally projecting PVN neurons in OZRs treated with scrambled shRNA, an effect that was eliminated in OZRs treated with AAV-OXR1-shRNA (n=15 neurons in each group). **P*<0.05 compared with the basal values in each group; #*P*<0.05 compared with respective values in OZRs treated with scrambled shRNA. AAV indicates adeno-associated virus; OXR1, orexin receptor 1; OZRs, obese Zucker rats; OZRs-SCM indicates OZRs treated with AAV-scrambled shRNA; OZRs-shRNA, OZRs treated with AAV-OXR1-shRNA; SB, SB334867; shRNA, short hairpin RNA.

suggesting that this treatment specifically silences OXR1 rather than OXR2 expression. Previous studies have demonstrated that in different brain regions both OXR1 and OXR2 are involved in the regulation of blood pressure and sympathetic outflow. Although activation of OXR2 in the rostral ventrolateral medulla[41,42] or the nucleus tractus solitarius[43] induces depressor response, OXR2 in the PVN is not involved in the regulation of blood pressure or sympathetic outflow.[13]

The salient finding of this study was that silencing of OXR1 expression in the PVN decreased elevation of arterial blood pressure in OZRs. Researchers showed that knockout of genes encoding orexins or ablation of orexin neurons in the hypothalamus substantially decreased the basal blood pressure in nonobese rats[25,44] and that this depressor effect was attributable to a decrease in sympathetic vasomotor tone.[25] These findings suggest that the orexin system is tonically activated to maintain blood pressure and sympathetic outflow under physiological conditions. Another study demonstrated that although prepro-orexin mRNA expression was considerably decreased in the hypothalamus in OZRs, the OXR1 expression level was 4- to 6-fold higher in OZRs than in lean Zucker rats.[45] Together with findings showing that antagonism of OXR1 activity or silencing of OXR1 expression in the PVN decreases ABP in OZRs, these findings suggest that OXR1 likely is tonically activated in the PVN in OZRs and is critically involved in maintaining high ABP in this rat model of obesity. In high-fat-diet–induced obesity, it seems that orexin receptor-2 (OX2R), rather than OX1R, signaling mediates this phenotype of obesity.[46] In this regard, chronic central administration of an OX2R-selective agonist prevents diet-induced obesity.[46] However, it is not clear if OX2R mediates high blood pressure and elevated sympathetic vasomotor tone in high-fat diet-induced obesity.

In addition to decreasing ABP, silencing of OXR1 expression in the PVN rebalanced sympathovagal activity, as treatment with AAV-OXR1-shRNA decreased the LF domain

but increased the HF domain of the HRV spectrum in OZRs. To confirm that silencing of OXR1 expression in the PVN reduces sympathetic outflow, we directly assessed sympathetic outflow by measuring the RSNA in anesthetized OZRs. We found that silencing of OXR1 in the PVN not only reduced the baseline ABP and RSNA but also largely attenuated the sympathoexcitatory response induced by microinjection of orexin A into the PVN. Furthermore, treatment with AAV-OXR1-shRNA eliminated the sympathoinhibitory response induced by microinjection of the OXR1 antagonist SB334867 into the PVN in OZRs. These data suggested that downregulation of OXR1 expression in the PVN decreases elevated sympathetic drive and ABP in OZRs.

We found that treatment with AAV-OXR1-shRNA did not have a significant effect on weight gain in the OZRs compared with OZRs treated with scrambled shRNA. OXR1 protein expression in the PVN in OZRs is increased at 4 to 6 weeks of age, when OZRs are not obese, and significantly increased after 4 to 6 weeks of age, when they quickly gain weight.[13,47] Thus, upregulation of OXR1 expression in the PVN seems to result from obesity rather than mutation of leptin receptors in OZRs. Plasma leptin levels are positively correlated with body fat.[48] However, whether elevated plasma leptin levels lead to upregulation of OXR1 expression in the PVN in OZRs is unclear. On the other hand, upregulation of OXR1 expression in the PVN is one of the factors involved in elevated sympathetic vasomotor tone in OZRs.[13] Thus, downregulation of OXR1 expression in the PVN reduces sympathetic vasomotor tone but does not affect body weight gain in OZRs.

Previous studies have shown that the neuronal activity of PVN presympathetic neurons crucially regulates sympathetic outflow.[13,36] Thus, we sought to determine whether treatment with AAV-OXR1-shRNA decreases PVN presympathetic neuron activity in OZRs. We found that silencing of OXR1 expression in the PVN markedly decreased the basal activity of spinally projecting PVN neurons in OZRs. Because PVN presympathetic neurons are major excitatory driving sources that lead to elevated sympathetic outflow under physiological and disease conditions such as hypertension and obesity,[17,39] these data suggest that OXR1 expression in the PVN was closely associated with the excitability of spinally projecting PVN neurons and sympathetic outflow in the OZRs. Consistent with a previous finding that OXR1 in the PVN mediates the excitatory effect of orexin A on spinally projecting PVN neurons,[13] we found that silencing of OXR1 in the PVN abolished the stimulatory effect of orexin A on spinally projecting neurons. A previous study has shown that overexpression of inwardly rectifying K^+ channels in the PVN induces sustained depressor response in hypertensive rats.[17] Although the researchers did not examine the activity of PVN presympathetic neurons, overexpression of inwardly rectifying K^+ channels likely depressed the activity of neurons within the PVN, including PVN presympathetic neurons, because a nonselective neuronal promoter was used to drive this overexpression. Also, studies demonstrated that inhibitory GABAergic synaptic inputs are the majority of local synaptic inputs to PVN neurons.[49,50] The K^+ channels overexpressed in these inhibitory PVN neurons may attenuate the observed depressor response in hypertensive rats. Thus, the role of PVN neurons in mediating the depressor effect in the study described above may have been underestimated. In the present study, treatment with AAV-OXR1-shRNA largely reduced the upregulated OXR1 expression in PVN neurons, including spinally projecting PVN neurons, in OZRs. Thus, silencing OXR1 expression in the PVN most likely decreases ABP and sympathetic outflow through suppression of the heightened activity of spinally projecting PVN neurons.

In summary, our study demonstrated that chronic downregulation of OXR1 expression in the PVN decreased the activity of spinally projecting PVN neurons and reduced ABP and sympathetic outflow in OZRs. This finding provides a rationale for development of novel therapeutics targeting hypothalamic OXR1 to treat obesity-related hypertension.

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Disclosures

None.

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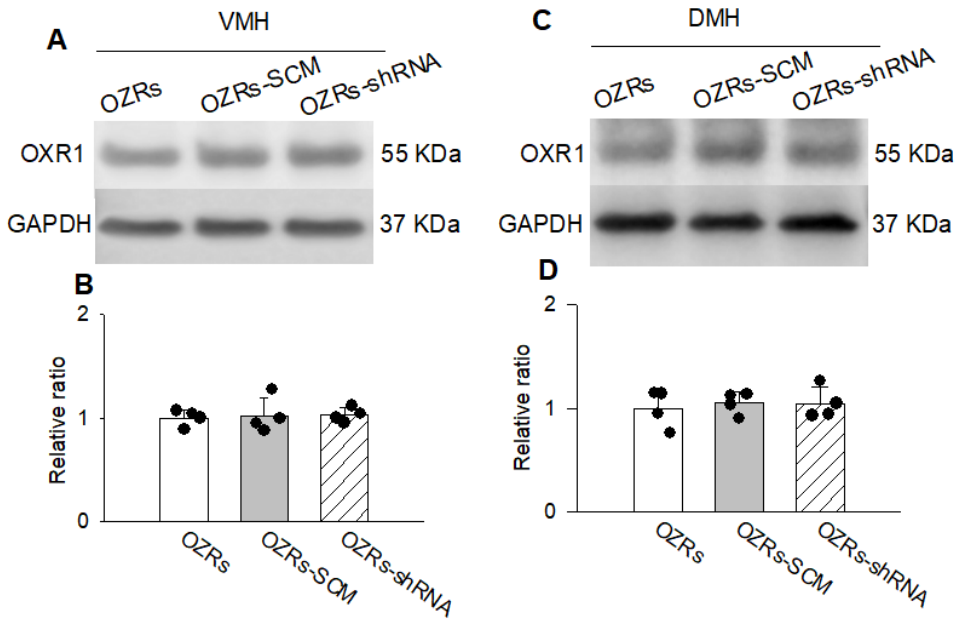
SUPPLEMENTAL MATERIAL

Figure S1. Silencing of OXR1 expression in the PVN did not affect the OXR1 protein level in the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH) in the OZRs.



Representative western blots and summary data showing that OXR1 protein levels in the VMH (*A* and *B*) and DMH (*C* and *D*) were not significantly changed in the OZRs treated with AAV-OXR1-shRNA compared with OZRs treated with the scrambled shRNA and the untreated OZRs. (n = 4 rats in each group). OZRs-SCM: OZRs treated with AAV-scrambled shRNA; OZRs-shRNA: OZRs treated with AAV-OXR1-shRNA.