Neuroanatomical organization and functional roles of PVN MC4R pathways in physiological and behavioral regulations

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ABSTRACT

Objective: The paraventricular nucleus of hypothalamus (PVN), an integrative center in the brain, orchestrates a wide range of physiological and behavioral responses. While the PVN melanocortin 4 receptor (MC4R) signaling (PVNMC4R+) is involved in feeding regulation, the neuroanatomical organization of PVNMC4R+ connectivity and its role in other physiological regulations are incompletely understood. Here we aimed to better characterize the input-output organization of PVNMC4R+ neurons and test their physiological functions beyond feeding.

Methods: Using a combination of viral tools, we mapped PVNMC4R+ circuits and tested the effects of chemogenetic activation of PVNMC4R+ neurons on thermoregulation, cardiovascular control, and other behavioral responses beyond feeding.

Results: We found that PVNMC4R+ neurons innervate many different brain regions that are known to be important not only for feeding but also for neuroendocrine and autonomic control of thermoregulation and cardiovascular function, including but not limited to the preoptic area, median eminence, parabrachial nucleus, pre-locus coeruleus, nucleus of solitary tract, ventrolateral medulla, and thoracic spinal cord. Contrary to these broad efferent projections, PVNMC4R+ neurons receive monosynaptic inputs mainly from other hypothalamic nuclei (preoptic area, arcuate and dorsomedial hypothalamic nuclei, supraoptic nucleus, and pre mammillary nucleus), the circumventricular organs (subfornical organ and vascular organ of lamina terminalis), the bed nucleus of stria terminals, and the parabrachial nucleus. Consistent with their broad efferent projections, chemogenetic activation of PVNMC4R+ neurons not only suppressed feeding but also led to an apparent increase in heart rate, blood pressure, and brown adipose tissue temperature. These physiological changes accompanied acute transient hyperactivity followed by hypovacuity and resting-like behavior.

Conclusions: Our results elucidate the neuroanatomical organization of PVNMC4R+ circuits and shed new light on the roles of PVNMC4R+ pathways in autonomic control of thermoregulation, cardiovascular function, and biphasic behavioral activation.

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Keywords Melanocortin 4 receptor; Paraventricular nucleus of hypothalamus; Blood pressure; Thermoregulation; Sympathetic nervous activity

1. INTRODUCTION

Obesity is a major risk factor for the development of hypertension; several population studies indicate that at least two thirds of the prevalence of hypertension can be directly attributed to obesity [1]. Sympathetic overactivity has been established in obese individuals and is considered one of the major contributors to the development of obesity-associated hypertension [2,3]. Mounting evidence indicates that the central melanocortin signaling pathway, mainly via melanocortin 4 receptor (MC4R), plays a key role in obesity [4–7]. Besides having a prominent role in energy homeostasis [8,9], MC4R signaling pathways are known to affect the sympathetic activity and cardiovascular physiology. Central administration of MC4R agonist steadily increases blood pressure (BP) and sympathetic traffic to various organs, including the kidney [10–12]. In contrast to the elevated sympathetic tone and BP commonly observed in obesity, morbidly obese humans and rodents due to monogenic MC4R-deficiency exhibit low to normal sympathetic nerve activity (SNA) and BP [13–17], suggesting a required but dissociable role of MC4R signaling in both metabolic and cardiovascular regulations in the context of obesity. Notably, accumulating evidence indicates the need for MC4R for leptin-induced sympathoexcitation; both pharmacological and genetic blockade of
MC4R abolish the leptin-mediated increase in sympathetic traffic to the kidney [11,12,18]. Moreover, central infusion of MC4R antagonist significantly decreases BP even in spontaneously hypertensive rats with normal body weight [17], thereby confirming the vital role of the MC4R signaling pathway in neurogenic hypertension beyond obesity. While the above studies underscore physiologically important roles for brain MC4R signaling in sympathetic activity and BP regulation, the neural basis underlying MC4R regulation of sympathetic control of cardiovascular function remains poorly understood. The paraventricular nucleus of hypothalamus (PVN), an indispensable integrative center in the brain, coordinates numerous physiological and behavioral responses, such as feeding, metabolic homeostasis, and sympathetic control of cardiovascular function, via its connections to diverse brain regions including different brainstem nuclei and spinal cord [19]. Importantly, the PVN has the highest expression of MC4R among other hypothalamic and brainstem nuclei [20,21]; PVN MC4R signaling has been well known for its role in the regulation of feeding and body weight homeostasis [22–24]. Additionally, a pharmacological study has shown that PVN MC4R signaling may also affect the sympathetic activity and BP regulation. Microinjection of MC4R agonist directly into the PVN of anesthetized mice increases BP and sympathetic traffic to the kidney through canonical MC4R-GzscAMP-PKA pathway [25]. This finding was supported by another study that showed that the ability of systemic MC4R agonist to increase BP is lost in mice lacking Gzsc in entire PVN neurons [26]. Remarkably, a slice electrophysiological study has shown that MC4R agonist increases the firing of the rostral ventrolateral medulla (RVLM)-projecting pre-synaptic PVN neurons in Zucker rats [27], with a greater increase of firing activity in obese rats compared to lean controls. This suggests that enhanced activity of PVN MC4R signaling pathway may underlie obesity-associated hypertension. While these pharmacological and ex vivo electrophysiological approaches support a role for PVN MC4R signaling in sympathetic control of cardiovascular function, the neuroanatomical organization of PVN MC4R pathways and the effects of selective activation of PVN MC4R neurons on cardiovascular and other behavioral and physiological parameters remains unclear.

In the present study, we mapped PVN MC4R circuits using a combination of viral tools and tested the hypothesis that indiscriminate activation of PVN MC4R neurons not only affects feeding but also other behavioral physiological parameters through their diffuse projections to brain regions associated with autonomic and behavioral responses.

2. METHODS

2.1. Animals

MC4R-2a-Cre knock-in mice (Jackson Stock No: 030759) were obtained from Dr. Brad Lowell group [24]. Mice were group-housed in the University of Iowa’s vivarium in a temperature-controlled environment (lights on 06:00, Light off 18:00) with free access to standard chow diet and water. Mice used in the present study were all maintained in C57BL/6 and 129 mixed backgrounds. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Iowa; and are as per NIH guidelines for the use and care of Laboratory Animals.

2.2. Viral vectors for neuronal tracing

Viral vectors from different scientific vendors were used to examine the neuroanatomical organization and the functional roles of PVN MC4R circuits, including Cre-dependent AAV2-DIO-Chr2-eYFP (Addgene), AAV2-DIO-mCherry (Addgene), AAV2-DIO-hM3Delta-mCherry (Addgene), AAV-retro-DIO-Flp (Duke Viral Vector Core); Flp-dependent AAV8-fDIO-TVA-mCherry (Salk Institute Viral Vector Core) and AAV8-fDIO-G (Salk Institute Viral Vector Core); and glycoprotein-deleted rabies virus (RV-EnvA-ΔG-GFP, Salk Institute Viral Vector Core).

2.3. Stereotoxic surgery

Stereotoxic surgery was performed as previously described [28]. Briefly, male MC4R-Cre+ mice were deeply anesthetized by intraperitoneal (IP) injection of ketamine/xylazine (100:10 mg/kg) and placed on a Kopf stereotaxic apparatus (Tujunga, CA). Following standard disinfection procedure, ~1.0 cm incision was made to expose the skull of the mice and a small hole was drilled into the skull bilaterally at defined positions to target the PVN (coordinates: AP -0.8 mm, ML +/-1.1 mm, DV -4.9 mm with 10-degree injection arm). Pulled glass micropipette filled with a viral vector was slowly inserted to reach the targeted brain region and a small volume (150–200 nl) of injection was made by applying pulse pressure using Triteck pressure Microinjector (Tritech Research, Los Angeles, CA). After 10 min of waiting to ensure full penetration of the injectant into the targeted area, the needle was slowly removed and the incision closed by wound clips. Then, the mice were kept on a warming pad until awake and fed a regular chow diet throughout the experimental period unless otherwise required.

2.4. Fluorescent double immunohistochemistry (IHC)

Brains of all mice that received viral injection were processed for histological verification of correct stereotoxic targeting. Immunohistochemistry (IHC) was performed as previously reported [28,29]. Briefly, the mice were transcardially perfused with cold Phosphate-buffered saline (PBS) followed by 10% neutralized formalin; the brains were removed and immersed in 25% sucrose solution; brains were cut into five series of 30 μm sections, and then stored in cryoprotectant at −20 °C until processed for IHC. One series of brain sections were rinsed in PBS, blocked in 3% normal donkey serum and 0.3% Triton X-100 in PBS for 30 min at room temperature. The sections were then incubated with primary antibodies against GFP (Cat#: GFP-1020, Aves Labs), mCherry (Cat#: 632496, Clontech), or c-Fos (Cat#: PC38, Cal-BioChem) overnight at 4 °C, and then washed and incubated with Cy2- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch) for fluorescent visualization, as per the manufacturer’s protocols.

2.5. Neuroanatomical tracing of input—output organization of PVN MC4R+ neurons

For the mapping of efferent projections PVN MC4R+ neurons, adult MC4R-Cre+ male mice (12–20 weeks old) received a unilateral stereotoxic injection of AAV2-DIO-Chr2-eYFP (~150 nl) into the PVN using a glass micropipette pressure injection system, as described above. After 3–4 weeks of viral injection, the mice were transcardially perfused with 10% neutralized formalin, and the whole brains were cut into five series of 30 μm sections and then stored in cryoprotectant at −20 °C until further processed for IHC. Brain sections were processed for fluorescent IHC for eYFP (using chicken anti-GFP antibody, Cat#: GFP-1020, Aves Labs) and the sections were mounted onto gelatin-coated slides for microscopic imaging. The slides were imaged using a slide-scanning microscope (VS120, Olympus) and the images were captured and analyzed using the OlyVIA software. Only correctly targeted four cases with minimal contamination to adjacent brain regions were used for whole-brain manual qualitative evaluation of efferent projections. For whole-brain mapping of monosynaptic inputs to PVN MC4R+ neurons, a mixture of AAV-DIO-flop (Cre-dependent) and Flp-dependent AAV8-fDIO-TVA-mCherry and AAV8-fDIO-RG was unilaterally injected into
the PVN of MC4R-Cre+ mice using glass micropipette pressure injection system, as aforementioned. After three weeks of stereotoxic surgery, a small volume (~100 nl) of glycoprotein-deleted rabies virus (RV-EnvA-ΔG-GFP) was injected into the same side of PVN where a mixture of AAV was previously infused. One week post-RV-EnvA-ΔG-GFP injection, the mice were transcardially perfused with 10% neutralized formalin, and the whole brains were cut into five series of 30-μm sections and then stored in cryoprotectant at −20 °C until further processed for IHC. One series of brain sections from each brain were processed for fluorescent IHC for GFP and the sections were then mounted onto coated microscope slides for imaging. Slides were imaged by a slide-scanning microscope (VS120, Olympus) and the images were captured and analyzed using OlyVIA software. Only correctly targeted cases with minimal contamination to adjacent brain regions (n = 4) were used for the brain-wide manual qualitative evaluation of neurons sending monosynaptic inputs to PVNMC4R neurons.

2.6. Feeding and glucose homeostasis

The effect of designer receptor exclusively activated by designer drugs (DREADD) activation of PVNMC4R neurons on feeding and glucose homeostasis was evaluated in two independent cohorts of experimental mice. Cohort 1 had adult (10–16 weeks) MC4R-Cre+ male mice (n = 7) that received a stereotoxic injection of AAV2-DIO-hM3Dq-mCherry into the PVN. For feeding, ad lib-fed mice were divided into two groups and they received intraperitoneal (IP) injection of either saline or small molecule DREADD receptor agonist clozapine-N-oxide (CNO; 2 mg/kg) right before dark cycle (6 PM) and food consumption of pellets was continuously monitored for additional 24 h. After one week of recovery from the surgery, both groups of mice were subjected to radio-telemetry implantation (PA-C10, Data Science). The distance traveled within arena, body mobility, the time spent in food and shelter zones, the number of water licks, and wheel-running activity were quantified and compared between the groups. At the end of the experiment, mice were transcardially perfused with 10% neutralized formalin and brains were extracted for histological verification of stereotoxic targeting.

2.7. Body surface temperature

Cohort-1 experimental mice from the feeding study (n = 7) were subjected to infrared (IR) temperature measurement using a high-resolution infrared camera (A655sc Thermal Imager; FLIR Systems, Inc.) as described previously [31]. Again, a crossover design was used to evaluate the effects of PVNMC4R neuron activation on thermoregulation. Briefly, the mice were imaged on a multilane treadmill (without active shock grid turned on) during 3 min of slow walking (7 m/min and 15° incline), as published previously [32]. Images were captured at 1fps. Ten images were quantified for each time point using FLIR ResearchIR software (version 3.4.13039.1003) and intensity was averaged for making group comparison. After the measurement of baseline temperature, the mice received IP injection of either saline or CNO (2 mg/kg) and were then returned to their home cages. The body surface temperature was monitored again 1-h post-injection and the change in temperature from the baseline was determined and compared between the groups.

2.8. Core body temperature

Cohort-2 experimental mice from the feeding study were subjected to the measurement of core body temperature upon DREADD activation of PVNMC4R neurons. To this end, both groups of DREADD (n = 8) and control (n = 7) mice were implanted with temperature transponders (IPTT-300 HTEC, Bio Medic Data Systems) into the abdominal cavity. After one week of recovery from the surgery, both groups of mice were given IP injection of CNO (2 mg/kg) and the core body temperature was monitored every 10 min up to 90 min in their home cages using a Temperature Probe Reader (DAS-8027iUS, Bio Medic Data Systems). The change in core body temperature from the baseline (before CNO injection) was calculated for each mouse and compared between the groups.

2.9. Behavioral measurements in PhenoTyper cages

Cohort-1 experimental mice from the feeding study (n = 7) were subjected to behavior characterization using Model 3000 Noldus PhenoTyper chambers (Noldus, Wageningen, Netherlands), as earlier described [33]. PhenoTyper chamber has an infrared CCD camera installed on the top, which allows automated tracking of mouse movement throughout the experimental period. White shredded paper bedding was given on the floor to mimic a home cage-like environment. The arena floor (30 × 30 cm) was further divided into 4 subarenas, namely wheel running, food, water, and shelter zones (Figure 6A). Video tracking and quantification of mouse behaviors were performed using EthoVision XT 15 software (Noldus Information Technology). Mice were acclimated in the chambers overnight prior to the testing. A crossover design was used to evaluate behavioral changes in response to chemogenetic activation of PVNMC4R neurons. The distance traveled within arena, body mobility, the time spent in food and shelter zones, the number of water licks, and wheel-running activity were quantified and compared between the groups. At the end of the experiment, mice were transcardially perfused with 10% neutralized formalin and brains were extracted for histological verification of stereotoxic targeting.

2.10. Radio-telemeter implantation and BP measurements

Adult (10–16 weeks) MC4R-Cre+ male mice that received a stereotoxic injection of either AAV2-DIO-hM3Dq-mCherry (DREADD group, n = 6) or AAV2-DIO- mCherry (control group, n = 3) into the PVN were subjected to radio-telemetry implantation (PA-C10, Data Science
Instruments) for continuous BP and heart rate (HR) monitoring, as previously reported [34]. Briefly, animals were deeply anesthetized with ketamine/xylazine (100 mg/10 mg/kg) and then kept on a heating pad to ensure proper maintenance of body temperature. Topical application of eye ointment was given to minimize corneal desiccation. Radio-telemeters were disinfected in actril solution overnight and then kept in sterile saline prior to surgical implantation. Under aseptic surgical conditions, the catheter of the radio-telemeter was inserted into the left carotid artery and tied securely using a 6-0 surgical suture. The transmitter was tunneled subcutaneously from the neck until the unit reached the midabdominal region. The neck incision was sutured closed with 4-0 absorbable cat gut and then further sealed with tissue adhesive (Vet-Bond) along the incision line. Warmed sterile saline was kept in sterile saline prior to surgical implantation. Under aseptic surgical conditions, the catheter of the radio-telemeter was inserted into the left carotid artery and tied securely using a 6-0 surgical suture.

After about two weeks of recovery from the surgery, both mouse groups were subjected to the measurements of BP and HR in their home cages. Mice were handled and subjected to mock IP injection daily (at random times) for 4–5 days to minimize the confounding effects of handling and stress induced by IP injection. On the test day, the mice were given IP injection of CNO (2 mg/kg) in the morning (~8 AM) and then again in the evening right before the dark cycle (18:00) to evaluate circadian effects of DREADD activation of PVNMC4R+ neurons on cardiovascular function. BP was continuously monitored and analyzed by the PhysioTel CONNECT system and LabChart software (ADInstruments). At the end of the experiment, mice were transcardially perfused with 10% neutralized formalin and brains were extracted for histological verification of correct targeting of hM3Dq-mCherry in the PVN.

2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA) software. Comparisons were made by Student’s t-test, multiple t-tests, or two-way ANOVA with Holm-Sidak post-hoc analysis as previously noted. Value of P < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

3. RESULTS

3.1. PVNMC4R+ neurons broadly innervate brain regions involved in feeding, neuroendocrine regulation, and autonomic control

To understand the neuroanatomical organization of PVNMC4R+ pathways, we performed viral-mediated anterograde tract-tracing of PVNMC4R+ neurons. Unilateral microinjection of Cre-dependent AAV2-DIO-ChR2-eYFP was made into the PVN of MC4R-Cre mice (Figure 1A). After 3–4 weeks, mice were transcardially perfused with 10% formalin and brains were processed for histological verification. Imaging of eYFP immunoreactivity confirmed four precisely targeted cases with minimal transduction of neurons outside the PVN (Supplemental Figure 1). Subsequent evaluation of the distribution of eYFP fibers revealed that PVNMC4R+ neurons (Figure 1B) broadly innervate different brain regions involved in feeding, thermoregulation, neuroendocrine function and autonomic regulation, including but not limited to the bed nucleus of the stria terminalis (BNST), preoptic area (POA), lateral hypothalamic area (LHA), the dorsomedial nucleus of hypothalamus (DMH), ventromedial nucleus of hypothalamus (VMH), arcuate nucleus of hypothalamus (ARC), median eminence (ME), anterior paraventricular thalamic nucleus (PVA), periaqueductal gray (PAG), parabrachial nucleus (PBN), pre-lucus coeruleus (preLC), nucleus of solitary tract (NTS), the dorsal motor nucleus of the vagus (DMV), rostral and caudal ventrolateral medulla (RVLM and CVLVM), and thoracic spinal cord (TSC) (Figure 1C-Y). Qualitative visual estimation of relative eYFP fiber density across brain regions is summarized in Supplemental Table 1. Notably, the projections to most brain regions were largely unilateral (Supplemental Figure 2A–H), except PVA, NTS, and DMV where we observed a strong bilateral innervation of PVNMC4R+ neurons (Figure 1V and Supplemental Figure 2C and N).

3.2. Mapping of monosynaptic inputs to PVNMC4R+ neurons

After the whole brain mapping of afferent projections of PVNMC4R+ neurons, we aimed to determine the brain regions where neurons send monosynaptic inputs to PVNMC4R+ neurons. For this purpose, we injected a combination of AAVs driving the expression of TUA and glycoprotein (G) that are required for cell-type-specific monosynaptic inputs into the PVN (Figure 2A,B). After 3 weeks, EnvA-pseudotyped G-deleted Rabies viruses (RV-EnVA-G-GFP) were again injected into the PVN of the MC4R-Cre+ mouse to label neurons sending monosynaptic inputs to PVNMC4R+ neurons, as previously reported [36]. Contrary to broad efferent projections, we observed monosynaptic labeling (GFP+ neurons) in relatively limited brain regions (observations made from four successfully targeted cases), mainly other hypothalamic nuclei, including ARC, DMH, POA (MPO, MnPO, and LPO), SON, VMH, LHA, and PMN (Figure 2E–F, 2H–K). We also documented that neurons in two circumventricular organs, the subfornical organ (SFO) and organum vasculosum of the lamina terminalis (OVLT), make monosynaptic inputs to PVNMC4R+ neurons (Figure 2E,G). We observed a large number of GFP+ neurons in BNST and PBN (Figure 2D–F, 2N), and sparse GFP+ neurons in DRN, PAG, vSUB, and NTS (Figure 2L–M, O). Overall patterns of monosynaptic inputs to PVNMC4R+ neurons are summarized in the schematic shown in Figure 2P.

3.3. DREADD activation of PVNMC4R+ neurons suppress feeding but not glucose homeostasis

To evaluate the functional role of PVNMC4R+ neurons, hM3Dq-DREADD-mediated activation of PVNMC4R+ neurons was performed. Bilateral microinjection of Cre-dependent AAV-DIO-hM3Dq-mCherry into the PVN of MC4R-Cre+ mice resulted in activation of PVNMC4R+ neurons, as assessed by double fluorescent IHC for mCherry and e-Fos (Supplemental Figure 3A and B), an established marker of neuronal activation. After confirming the effective DREADD-mediated activation of PVNMC4R+ neurons, we first tested the effects of DREADD activation of PVNMC4R+ neurons on feeding and glucoregulation. As previously shown [24], DREADD activation of PVNMC4R+ neurons right before the onset of dark cycle significantly suppressed feeding (Supplemental Figure 3C), which was not observed when CNO was administered to control mice without hM3Dq-DREADD expression (Supplemental Figure 4A). Contrastively, neither baseline glucose nor glucose tolerance was affected by DREADD activation of PVNMC4R+ neurons (Supplemental Figure 3D–E). Importantly, these observations were supported in an independent experimental cohort of MC4R-Cre+ male mice that received either AAV2-DIO-hMS3q-mCherry (DREADD group, n = 8) or AAV2-DIO-mCherry (control group, n = 7) into the PVN. Effective activation (e-Fos) of PVNMC4R+ neurons was only observed in the DREADD group, but not the control group, upon IP injection of CNO (2 mg/kg) (Figure 3A–C). Continuous monitoring of food intake with FED3 attached to home cage revealed that IP injection of CNO right before dark phase significantly suppresses food intake in DREADD group compared to control group (Figure 3D). GTT with 15-min pretreatment of CNO in 5-hour fasted mice again revealed no effect of the DREADD activation of PVNMC4R+ neurons on systemic glucose homeostasis (Figure 3E).
3.4. DREADD activation of PVNMC4R⁺ neurons affect thermoregulation

In contrast to the well-established role in feeding regulation, the role of PVNMC4R⁺ pathways in autonomic activity-driven thermoregulation remains unclear. Based on the observation of broad innervation of PVNMC4R⁺ neurons to many brain regions involved in sympathetic activation and thermoregulation (Figure 1Y), we next tested whether PVNMC4R⁺ circuits also affect autonomic thermoregulation. To this end, mice were placed on the treadmill for slow walking and body surface temperature was monitored by a high-resolution IR thermal imaging camera as the baseline. Mice then sequentially received IP injection of saline and CNO (2 mg/kg) with 1-hour interval and body surface temperature was taken 1-hour post-injection to evaluate the changes of body surface temperature. While IP saline did not result in notable changes in temperature as expected, there was an obvious increase in body surface temperature after 1-hour IP CNO (Figure 4A). Quantification revealed that DREADD activation of PVNMC4R⁺ neurons significantly increases the temperature in the neck area (brown fat), low back and tail (Figure 4B). The changes in temperature of the neck area and low back were not observed when CNO was given to control mice without hM3Dq-DREADD expression; however, there was a slight but significant increase in tail temperature after CNO treatment in control mice (Supplemental Figure 4B). Literature indicates that increased tail temperature is an indication of vasodilation which occur in rodents as a mean to promote heat dissipation and reduce body temperature [37]. Therefore, DREADD activation of PVNMC4R⁺ neurons may have caused an increase in core body temperature, leading to a counter-regulatory heat-dissipating response by promoting vasodilation. To directly test this possibility, we implanted a temperature transponder into the abdominal cavity of both DREADD and control mice and monitored the core body temperature upon IP injection of CNO. As we predicted, DREADD activation of PVNMC4R⁺ neurons caused a slight but significant increase in core body temperature compared to control mice (Figure 4C).

3.5. DREADD activation of PVNMC4R⁺ neurons increase BP and HR

Neurons in the PVN are critical in determining the sympathetic tone to the cardiovascular system and thereby affect BP and HR. We observed dense projections of PVNMC4R⁺ neurons to brain regions that are important for cardiovascular control, including PAC, NTS, RVLM, and without hM3Dq-DREADD expression; however, there was a slight but significant increase in tail temperature after CNO treatment in control mice (Supplemental Figure 4B). Literature indicates that increased tail temperature is an indication of vasodilation which occur in rodents as a mean to promote heat dissipation and reduce body temperature [37]. Therefore, DREADD activation of PVNMC4R⁺ neurons may have caused an increase in core body temperature, leading to a counter-regulatory heat-dissipating response by promoting vasodilation. To directly test this possibility, we implanted a temperature transponder into the abdominal cavity of both DREADD and control mice and monitored the core body temperature upon IP injection of CNO. As we predicted, DREADD activation of PVNMC4R⁺ neurons caused a slight but significant increase in core body temperature compared to control mice (Figure 4C).
Figure 2: Monosynaptic inputs to PVNMC4R⁺ neurons. (A) Experimental timeline showing sequential viral injection (n = 4). (B) Schematic showing the viral strategy to map the neurons sending monosynaptic inputs to PVNMC4R⁺ neurons. (C) Representative images showing the successful targeting of PVN with different viral vectors. (D–O), Representative images showing GFP-positive cells observed throughout different brain regions. (P) Schematic depicting brain regions where neurons send monosynaptic inputs to PVNMC4R⁺ neurons. Note that the size of the filled circle in each brain region represents the relative optical density of observed GFP⁺ cells (smallest circle represents few scattered cells and the largest circle represents a dense group of cells). Abbreviations: median preoptic nucleus (MnPO), organum vasculosum of the lamina terminalis (OVLT), medial preoptic area (MPO), lateral preoptic nucleus (LPO), subfornical organ (SFO), supraoptic nucleus (SON), dorsal premammillary nucleus (PMD), ventral premammillary nucleus (PMV), ventral hippocampal CA1 (vCA1), ventral subiculum (vSUB). Scale bar: 50 μm for C, 100 μm for G, H and O, 200 μm for D, E, F, J, K, L, M, and N.

Figure 3: The effect of DREADD activation of PVNMC4R⁺ neurons on feeding and glucose homeostasis. (A) Schematic showing bilateral microinjection of Cre-dependent AAV driving expression of excitatory hM3Dq-DREADD receptor into the PVN of MC4R-Cre⁺ mouse. (B) Representative images showing mCherry and c-Fos immunoreactivity in the PVN of MC4R-Cre⁺ mice that received either AAV-DIO-mCherry or AAV-DIO-hM3Dq-mCherry (Scale bar: 100 μm). (C) Quantification of c-Fos⁺ PVN mCherry cells. (D) Decreased food pellets consumption (FED3) after IP injection of CNO at the beginning of dark cycle. (E) The effects of DREADD activation of PVNMC4R⁺ neurons on GTT (n = 7 for mCherry and n = 8 for hM3Dq). ***p < 0.001 by Student’s t-test (C and pellets consumed 5 h post CNO in D) or by two-way ANOVA (time course pellets consumption in D). Data are presented as mean ± SEM.
Therefore, we tested whether PVNMC4R⁺ neuron activation also alters BP and HR. To ascertain this, the mice were implanted with radio-telemeters for continuous monitoring of BP and HR. After two weeks of recovery from the surgery, mice were tested for their cardiovascular response to IP injection of either saline or CNO in both light and dark phases. Consistent with broad projections to various cardiovascular brain regions, DREADD activation of PVNMC4R⁺ neurons by a single dose of CNO (2 mg/kg) resulted in a sharp increase in MAP and HR in DREADD mice compared to control mice (Figure 5 A–D). These cardiovascular responses elicited by DREADD activation of PVNMC4R⁺ neurons were apparent in the light cycle but not the dark cycle. Further analysis revealed that DREADD activation of PVNMC4R⁺ neurons increased both systolic arterial pressure (SAP) and diastolic arterial pressure (DAP) in the light cycle (Figure 5E–H). Importantly, CNO administration in non-DREADD control mice did not affect BP and HR (Figure 5I–J).

3.6. Behavioral alterations by DREADD activation of PVNMC4R⁺ neurons

Because of the broad innervation of PVNMC4R⁺ neurons to diverse brain regions that are important for behavioral regulation (BNST, LHA, VTA, PAG, etc), we also tested whether activation of PVNMC4R⁺ neurons elicits other behavioral responses. To this end, we subjected mice to PhenoTyper cages which allow continuous monitoring of different mouse behaviors in an automated manner. After overnight acclimation in the PhenoTyper cages, mice were given IP injection of either saline or CNO right before dark cycle (17:30–18:00) and then again next morning (8:00–9:00) to determine behavioral alterations in both dark and light cycles. The floor was further marked for 4 sub-arenas representing wheel running, food, water, and shelter zones, respectively (Figure 6A–B), which enable the calculation of time spent in each zone. We found that DREADD activation of PVNMC4R⁺ neurons led to an initial transient (1-hour post-injection) increase followed by significant suppression of ambulatory activity and body mobility (Figure 6C–D). The mice also spent more time in the shelter zone but less time in the food zone upon DREADD activation of PVNMC4R⁺ neurons (Figure 6E–F). No alteration in water drinking behavior was observed (Figure 6G). Consistent with reduced ambulatory activity, voluntary wheel running activity was also significantly reduced upon DREADD activation of PVNMC4R⁺ neurons (Figure 6H).

4. DISCUSSION

The PVN is a key node in the brain orchestrating a wide range of physiological and behavioral responses, including but not limited to fear, fight-or-flight response, feeding, metabolic homeostasis, and autonomic regulation of cardiovascular function. PVNMC4R⁺ neurons represent a small subset of PVN neurons; they are largely distinct from many well-known PVN neurons expressing different neuropeptides [20,38]. Substantial evidence supports a role for PVNMC4R⁺ pathways in feeding regulation mainly via PVNMC4R⁺ → PBN circuit [23,24,39]. In the current study, we provide further evidence to show a rather complex role of PVNMC4R⁺ neurons beyond feeding. We observed a significant increase in body surface temperature around the neck and lower back upon DREADD activation of PVNMC4R⁺ neurons, indicating a likely elevation of sympathetic drive.
the BAT. This observation supports a previous finding that PVNMC4R+ neurons polysynaptically innervate the BAT [40,41], intra-PVN injection of MC4R agonist MTII increase BAT temperature [40], and selective restoration of MC4Rs in Sim-1 PVN injection of MC4R agonist MTII increase BAT temperature [40], and selective restoration of MC4Rs in Sim-1 PVN could be one of the important brain regions where MC4R signaling affects cardiovascular physiology. We observed a sharp increase in MAP and HR upon DREADD activation of PVNMC4R+ neurons, especially when neurons are activated in the light cycle, but not the dark cycle. The peak responses of BP and HR were even higher when activated in the light cycle compared to the dark cycle (Figure 5). The underlying mechanism of this light phase-specific rise in BP and HR by PVNMC4R+ neuron activation remains unclear but may involve circadian-specific responses of effector organs (vasculature and heart) to a sudden increase in sympathetic outflow. Our findings are consistent with previous pharmacological observations showing that intra-PVN injection of MTII increases MAP in anesthetized lean Zucker rats [25] and that MTII-mediated increase in BP is lost in mice lacking Grxs in PVN neurons [26], arguing a plausible role of PVNMC4R signaling in sympathetic control of cardiovascular function. Behavioral monitoring in Phenotype cages unexpectedly revealed that DREADD activation of PVNMC4R+ neurons acutely increases ambulatory movements followed by an obvious behavioral suppression, including reduced locomotor activity, feeding and voluntary wheel running. These behavioral suppressions indicate that increase in BP and HR is unlikely secondary to the behavioral alterations, implying there could be segregated PVNMC4R+ pathways that differentially mediate behavioral and autonomic responses. Indeed, our anterograde tract-tracing revealed that PVNMC4R+ neurons broadly innervate different brain regions known to mediate different behavioral and physiological responses. Because of the well-known cardiovascular side effects of pharmacological activation of MC4R signaling, it is of significant interest for future studies to test whether there are anatomically distinct subsets of PVNMC4R+ neurons that differentially regulate feeding behavior and sympathetic control of cardiovascular function. For instance, several studies have shown that the PVNMC4R+→PBN and PVNMC4R+→pre-LC pathways can reduce feeding [23,24,39], however, whether these pathways also affect cardiovascular function is unknown. Our anterograde tracing clearly shows that in addition to the PBN, PVNMC4R+ neurons also heavily innervate brain regions involved in sympathetic control of cardiovascular function, including NTS, VML,
PAG, and TSC. However, the functional roles of these projections in the sympathetic control of cardiovascular function have not yet been explored. Another important question that arose from the present study is whether PVNMC4R$^+$ neurons send collateral projections to different brain regions to simultaneously affect both feeding and autonomic control of cardiovascular function and thermoregulation. More refined functional circuit mapping studies are needed to answer these important yet unresolved questions in the future.

There are some limitations of the current study. Although viral-mediated axonal tracing is widely used nowadays to delineate cell-type-specific brain circuits, whether most ChR2-eYFP fibers we observed across the brain regions make functional synaptic connections remains unclear. It is possible that some ChR2-eYFP fibers in certain brain regions could be just passing-through axons. Comprehensive ChR2-assisted circuit mapping with electrophysiological recording in those projected brain regions is required to prove the likelihood of true synaptic connections in future studies. Additionally, it is challenging to robustly cover the entire rostral-to-caudal structure of PVN without contaminating adjacent brain regions by stereotaxic injection; therefore, neuroanatomical mapping of both efferent and afferent circuits we presented here could have underestimated the actual input-output organization of PVNMC4R$^+$ neurons. Another potential concern is the use of CNO as an option for neuronal activation. Studies have shown that when used in high dose (>5 mg/kg), CNO can be reverse-metabolized into clozapine [43,44], an antipsychotic medication that could impact some behaviors and physiological measurements. While we did not observe significant effects of CNO on feeding, BAT temperature, and cardiovascular parameters in control mice, the tail temperature was found slightly but significantly increased by CNO treatment in control mice (although the effect was much smaller than that of DREADD activation of PVNMC4R$^+$ neurons). Since clozapine has been shown to have a vasodilatory effect [45,46], we speculate that this small increase of tail temperature could be due to vasodilation caused by clozapine that converted from CNO. It is therefore worthwhile to test in the future whether optogenetic activation of PVNMC4R$^+$ neurons can replicate some of the functional observations made in the present study.

5. CONCLUSION

In summary, we mapped the neuroanatomical connections of PVNMC4R$^+$ neurons and present further evidence to show that PVNMC4R$^+$ circuits mediate complex behavioral and physiological regulations beyond feeding. PVNMC4R$^+$ signaling pathways have been the focus of appetite suppression for potential anti-obesity therapeutics. Understanding the neural basis of PVNMC4R$^+$ signaling pathways for these additional behavioral and physiological regulations may therefore help to refine the strategy for targeting PVNMC4R$^+$ to treat obesity and associated complications, such as obesity-associated hypertension.
US and HC contributed to conceptualization, study design, methodology, and manuscript writing. US, JJ, KS, BAT, JED, SRR, YD, GD, BX, ZZ, JC6, and LVZ conducted and/or provided critical technical assistance for the experiments. US and HC contributed to the data analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101401.

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